



Assessment of *in vitro* digestion of reduced sugar biscuits with extruded brewers' spent grain

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ABSTRACT

This study aimed to evaluate the nutritional value and potential health claims for reduced sugar biscuits containing extruded brewers' spent grain (EBSG) and fructooligosaccharides (FOS). One traditional biscuit with added sugar and three reduced sugar biscuits containing 15.2 % FOS and EBSG (0, 8 and 17 %), with nutrition claims "high in fiber" and "source of protein" for those containing 17 % of EBSG, were formulated. Biscuits were characterized by analysis of nutrients and bioactive compounds before and after digestion under *in vitro* enzymatic oral-gastro-intestinal and colonic fermenting conditions. The bioaccessibility of antioxidants, anti-inflammatory and antidiabetic compounds in biscuits' intestinal digests and short-chain fatty acids in colonic samples was analyzed. EBSG-added biscuits showed significantly lower ($p < 0.05$) glucose intestinal bioaccessibility and significantly higher ($p < 0.05$) phenolic compounds intestinal bioaccessibility compared to biscuits without EBSG. EBSG-added biscuits showed significantly ($p < 0.05$) higher *in vitro* antidiabetic potential than the other did. Moreover, the intestinal digest of biscuits containing 17 % EBSG exhibited significantly ($p < 0.05$) better *in vitro* inhibition of intracellular ROS formation and *in vitro* anti-inflammatory properties. FOS addition ($p < 0.05$) significantly improved the production of butyric acid while EBSG did for valeric acid which possess chemoprotective effect. In conclusion, the combined use of FOS (15.2 %) and EBSG (17 %) allowed obtaining a human healthier snack formulation for satisfying consumers' demands and achieving nutrition security.

1. Introduction

Prevalence of non-communicable diseases such as obesity, diabetes, metabolic syndrome and cancer, is linked to low quality diets. These diets include energy dense foods high in low-quality fat and sugar and low in dietary fiber and proteins (Global Panel on Agriculture and Food Systems for Nutrition, 2016). Regarding the carbohydrates intake, in

2015 the World Health Organization recommended to reduce sugar intake to 5 % of the total energy intake (World Health Organization (WHO) (2015)). Although many diseases of public health significance can be prevented or treated by increasing the amount and variety of dietary fiber in food, actual dietary fiber intake is below recommended standards: 30–35 g/day for men and 25–32 g/day for women (Barber, Kabisch, Pfeiffer, & Weickert, 2020; Timm & Kagaku, 2018).

Abbreviations: BSG, Brewers' spent grain; EBSG, Extruded brewers' spent grain; TB, Traditional biscuit; FOS-EBSG0, Reduced sugar biscuit with 15.2% fructooligosaccharides and 0% extruded brewers' spent grain; FOS-EBSG8, Reduced sugar biscuit with 15.2% fructooligosaccharides and 8% extruded brewers' spent grain; FOS-EBSG17, Reduced sugar biscuit with 15.2% fructooligosaccharides and 17% extruded brewers' spent grain; D-TB, Intestinal digest of traditional biscuit; D-FOS-EBSG0, Intestinal digest of reduced sugar biscuit with 15.2% fructooligosaccharides and 0% extruded brewers' spent grain; D-FOS-EBSG8, Intestinal digest of reduced sugar biscuit with 15.2% fructooligosaccharides and 8% extruded brewers' spent grain; D-FOS-EBSG17, Intestinal digest of reduced sugar biscuit with 15.2% fructooligosaccharides and 17% extruded brewers' spent grain; F-TB, Colonic fermented digest of traditional biscuit; F-FOS-EBSG0, Colonic fermented digest of reduced sugar biscuit with 15.2% fructooligosaccharides and 0% extruded brewers' spent grain; F-FOS-EBSG8, Colonic fermented digest of reduced sugar biscuit with 15.2% fructooligosaccharides and 8% extruded brewers' spent grain; F-FOS-EBSG17, Colonic fermented digest of reduced sugar biscuit with 15.2% fructooligosaccharides and 17% extruded brewers' spent grain.

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Nowadays, consumers are interested in maintaining their health, looking for products that enhance their: immune support, digestive health and bone/joint health; and seeking for sustainable practices in the foods and beverages they purchase (Kerry, 2021), especially after COVID-19 pandemic (Wachyuni, Mada, & Wiweka, 2020).

Therefore, developing healthier and sustainable convenience foods is necessary to improve dietary habits, attend consumer demands and contribute to nutrition security. Biscuits have the potential to meet all these requirements. They are a widely consumed snack, which leads the fast-moving consumer goods category and whose worldwide market is expected to be USD 164 billion by 2024 (Goubgou et al., 2021). The sources of sugars in the European diet are soft drinks and sweet snacks including biscuits. Their formulation can be improved reducing their sugar content using different strategies including replacement of sugar with an alternative ingredient (WHO, 2020).

Brewers' spent grain (BSG) is the major brewing by-product and is a source of dietary fiber, protein and bioactive compounds, while it has a low content of digestible starch and fat (Lynch, Steffen, & Arendt, 2016). It is being commercialized as baking mixes, pasta, puffs and bars by the company ReGrained (<https://www.regrained.com/>) and also as flour by the organizations Grainstone (<https://www.grainstone.com.au/>) and Rise (<https://www.riseproducts.co/>). Previous studies have incorporated BSG into different food products such as pasta, snacks and biscuits and assessed its contribution to final product nutritional and functional quality (Farcas et al., 2021; Heredia-Sandoval et al., 2019; Ktenioudaki, Chaurin, Reis, & Gallagher, 2013; Petrović, Pajin, Tanackov, Pejin, & Aleksandar, 2015; Schettino et al., 2021). The impact of extruded BSG in food products has been published in previous studies (Ackar et al., 2018; Nascimento, Calado, & Carvalho, 2017; Reis & Abu-ghannam, 2014; Sobukola, Babajide, & Ogunsade, 2012; Steinmacher, Honna, Gasparotto, Anibal, & Grossmann, 2012) and has been summarized in a review paper (Naibaho, 2021).

In terms of its nutritional and functional quality as food ingredient, previous studies have analyzed the biological activity of BSG's dietary fiber (Fu, Yu, Li, Liu, & Li, 2011; Niemi et al., 2012; Zhang, Cao, Yin, & Wang, 2018; Zielke et al., 2017). In addition, the positive effect extrusion has on BSG's *in vitro* antioxidant, anti-inflammatory and antidiabetic properties has previously been determined (Gutierrez-Barrutia, Cozzano, Arcia, & Dolores, 2022). To the best of our knowledge, no articles on the bioaccessibility of nutrients and bioactive compounds of foods formulated using as ingredient extruded BSG (EBSG) are available. Thus, the study of its feasibility as food ingredient for sustainable healthy human diets, contributing to reduce the prevalence of NCDs, specifically those associated with metabolic disorders is of great interest.

Fructooligosaccharides (FOS) are prebiotic soluble dietary fiber consisting of linear chains of fructose units linked by β (1,2) glycosidic bonds (Sabater-Molina, Larqué, Torrella, & Zamora, 2009). FOS has low sweetness intensity that may range from 30 % to 50 % of that of sucrose what makes them useful for various kinds of foods where the use of sucrose is restricted, it has lower caloric value than digestible carbohydrates (1.5 kcal/g) and is not cariogenic (Abdul Rahim, Saeed, Khalid, Hussain, & Anjum, 2011; Sabater-Molina et al., 2009). FOS potential physiological effects include the immune system modulation by their prebiotic behavior which stimulates the growth of *Bifidobacterium* and by the production of short-chain fatty acids with immunomodulator and anti-inflammatory properties during colonic fermentation, as well as the reduction of serum lipids and glucose peaks in blood (Sabater-Molina et al., 2009), what goes in line with latest consumers demands. Moreover, EFSA has established a cause-effect between the formulation of foods and beverages with FOS instead of sugar and the reduction of postprandial glycemic response health claim (EFSA, 2014).

The aim of this study was to provide novel information on nutrition and potential health claims for an innovative reduced sugar biscuit containing as ingredients extruded brewers' spent grain (EBSG) and fructooligosaccharides. Therefore, biscuits nutrients and bioactive compounds content were determined. An *in vitro* oral-gastro-intestinal

digestion and colonic fermentation was done to assess the effect food matrix may have on its nutrients and bioactive compounds bioaccessibility and *in vitro* health promoting properties.

2. Materials and methods

2.1. Materials

Chemicals used were of reagent grade. Enzymes used for *in vitro* oral-gastro-intestinal digestion (α -amylase from human saliva-A0521, porcine gastric pepsin-P6887, porcine pancreatin-P1625, bile porcine extract-FOS-EBSG8631), Folin reagent, 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), fluorescein (FL) disodium salt, 2,20-azobis (2-methylpropionamide) dihydrochloride (AAPH), O-phthalaldehyde, were bought from Sigma-Aldrich (St. Louis, MO, USA). Glucose oxidase-peroxidase kit was from Spinreact (Girona, Spain). Quinine sulphate dehydrated was purchased from Thermo Scientific™ (Waltham, Massachusetts, USA). Megazyme (Dublin, Ireland) reagents were used for total dietary fiber (K-TDFR-200A) and total starch (K-RSTAR). Ferulic acid was from Honeywell Fluka.

For cellular studies, Dulbecco's modified Eagle medium (DMEM), L-Glutamine, antibiotics (penicillin and streptomycin) and trypsin were from Gibco Laboratory (Invitrogen Co, Grand Island, NY, USA) and fetal bovine serum (FBS) was from Hyclone (GE Healthcare, Chicago, IL, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorofluorescein diacetate (DCFH-DA), ascorbic acid were sulfanilamide, N-1-(naphthyl)ethylenediamine-dihydrochloride, phosphoric acid, sodium nitrite and lipopolysaccharide from E. coli O55:B5 (LPS) were purchased in Sigma-Aldrich (St. Louis, MO, USA).

2.2. Food ingredient

Fábricas Nacionales de Cerveza (Minas, Uruguay) provided the brewers' spent grain (BSG). It was dried in a convection oven ($45\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$) until its moisture content was below 5 % and grounded in a laboratory mill (Retsch ZM 200) until 0.5 mm particle size was achieved. Extruded brewers' spent grain (EBSG) was obtained using a single screw extruder (Brabender Co Cordero E330) and the operating conditions were: 15.8 % of sample moisture content, 164.3 revolutions per minute (rpm) and $122.5\text{ }^{\circ}\text{C}$ (Gutiérrez-Barrutia, del Castillo, Arcia, & Cozzano, 2022). Extrudates were grounded to 0.5 mm and kept at $-20\text{ }^{\circ}\text{C}$ for further analysis. Fig. 1 summarizes the procedure for obtaining extruded brewers spent grain (EBSG) and EBSG biscuits, which were both done in duplicate.

2.3. Tested foods: Biscuits

By modifying a traditional added sugar biscuit (TB) three different biscuits were formulated. Biscuits' formulations are presented in Table 1. Biscuits were supplemented with two types of commercialized fructooligosaccharides (FOS): Orafiti® P95 and Orafiti® L95 (Orafiti®GR, Beneo, Belgium). Biscuits were formulated with 15.2 % of FOS and three different amounts of EBSG added substituting wheat flour: 0 g/100 g (FOS-EBSG0), 8 g/100 g (FOS-EBSG8) and 17 g/100 g (FOS-EBSG17). Thus, 14 % and 30 % of FOS-EBSG0's wheat flour content was reduced in FOS-EBSG8 and FOS-EBSG17, respectively (Table 1). EBSG's content was chosen to reach the nutrition claim high in dietary fiber according to MERCOSUR/GMC/RES N° 01/12 legislation.

After preparing the dough, it was left to stand at $7\text{ }^{\circ}\text{C}$ for 24 h. Then it was manually stretched with a rolling pin to 0.3 cm and cut with a circular metallic cutter (6 cm diameter). Biscuits were baked in a convection oven for 15 min at $180\text{ }^{\circ}\text{C}$ and left to cool at room temperature. Finally, they were milled and kept frozen ($-20\text{ }^{\circ}\text{C}$) until further analysis.

To achieve the aim of the present study the methodology summarized in Fig. 2 was followed.

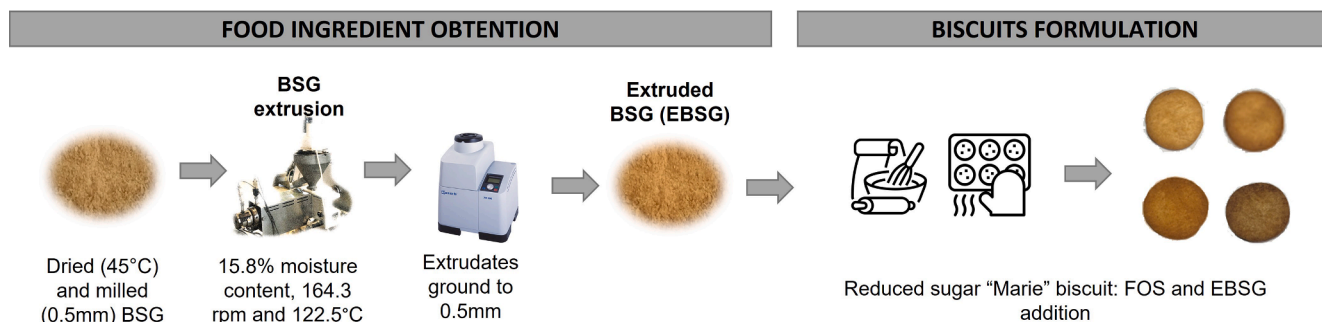


Fig. 1. Extruded brewers' spent grain (EBSG) and its application as ingredient in reduced sugar biscuit formulation. FOS: Fructooligosaccharides.

Table 1
Extruded brewers' spent grain biscuits formulation.

Ingredients	TB (g/100 g)	FOS-EBSG0 (g/100 g)	FOS-EBSG8 (g/100 g)	FOS-EBSG17 (g/100 g)
Wheat flour	54.0	57.0	49.0	40.0
EBSG	0.0	0.0	8.0	17.0
White sucrose	11.1	0.0	0.0	0.0
Brown sucrose	5.2	3.8	3.8	3.8
FOS (Orafti® P95)	0.0	10.2	10.2	10.2
FOS (Orafti® L95)	0.0	5.0	5.0	5.0
Baking powder	1.0	1.0	1.0	1.0
Butter	16.7	11.0	11.0	11.0
Egg	12.0	12.0	12.0	12.0
Sugar added*	16.3	4.9	4.9	4.9

EBSG: Extruded brewers' spent grain. FOS: fructooligosaccharides. TB: traditional biscuit. FOS-EBSG0: reduced sugar biscuit with no extruded brewers' spent grain. FOS-EBSG8: reduced sugar biscuit with 8 % extruded brewers' spent grain. FOS-EBSG17: reduced sugar biscuit with 17 % extruded brewers' spent grain.

* The sugar added to the biscuits was calculated as the sum of total amount of white and brown sucrose and FOS according to product specification sheet (8 % for Orafti® P95 and 6 % for Orafti® L95) added to the biscuits.

2.4. *In vitro* oral-gastro-intestinal digestion

All biscuits were subjected to *in vitro* oral-gastro-intestinal digestion according to Hollebeek et al (Hollebeek, Borlon, Schneider, Larondelle, & Rogez, 2013) modified by Martinez-Saez et al (Martinez-Saez, Hochkogler, Somoza, & del Castillo, 2017). The digests were centrifuged at 10,000 rpm, 4 °C for 20 min. Undigested fraction obtained at this stage were kept at -80 °C for further analysis. Supernatants were cleaned up by taking out bile salts. Finally, the soluble fraction containing duodenal bioaccessible compounds was recovered for further analysis (D-TB, D-FOS-EBSG0, D-FOS-EBSG8 and D-FOS-EBSG17). Experiments were performed in triplicate. Controls with the addition of inactivated enzymes were carried out.

2.5. *In vitro* simulation of colonic fermentation

Undigested fraction obtained in Section 2.4 were subjected to an *in vitro* simulation of gut fermentation procedure which was established based in several studies (Navajas-Porras, Pérez-Burillo, Valverde-Moya, & Hinojosa-Nogueira, 2021; Pérez-Burillo, Hinojosa-Nogueira, Pastoriza, & Rufián-Henares, 2020; Pérez-Burillo, Rufián-Henares, & Pastoriza, 2018) as follows. Fecal material was obtained from seven healthy volunteers (one man and six women) who have not received antibiotics for the last three months. For sample collection, volunteers were provided with sterile containers, BD GasPak™ EZ Anaerobe Container System Sachets and two zip bags. Fecal samples were gathered with a minimum of 16 h prior to analysis and remained at 4 °C under anaerobic

conditions until analysis. Experiments were done in an anaerobic cabin (BACTRON Anaerobic Environmental Chamber, SHELLAB, USA). Equal parts of fecal material from each volunteer were mixed with phosphate buffer (0.1 M, pH 7) at 30 % w/v. The fecal inoculum was homogenized using a stomacher (Stomacher 400 Circulator, SEWARD, U.K.) for 10 min. Fermentation medium consisted of peptone (15 g/L), cysteine (0.312 mg/L) and sodium sulfide (0.312 mg/L) and pH was adjusted to 7. Five hundred milligrams of undigested fractions obtained in Section 2.4 were mixed with 7.5 mL of fermentation medium and 2 mL of fecal inoculum in a screw tap tube. They were left under constant agitation for 24 h at 37 °C. Microbial growth was stopped by immersion in ice. Screw tap tubes were centrifuged at 4500 rpm for 10 min and supernatants were filtered (0.2 µm), which corresponded to the biscuits' colonic fermented digests (F-TB, F-FOS-EBSG0, F-FOS-EBSG8 and F-FOS-EBSG17). Colonic fermented digests were kept at -20 °C until further analysis. Experiments were done in triplicate for each sample.

2.6. Nutritional value assessment

2.6.1. Carbohydrates

2.6.1.1. *Starch*. Total, digestible and resistant starch content of biscuits was assayed based on AOAC 2002.02 (AOAC, 2012). Analyses were performed in triplicate and results expressed as g in 100 g of sample.

2.6.1.2. *Free glucose*. The amount of free glucose in biscuits and their intestinal digests (D-TB, D-FOS-EBSG0, D-FOS-EBSG8, D-FOS-EBSG17) was determined. Biscuits' free glucose was extracted as described by Protonotariou et al (Protonotariou, Mandala, & Rosell, 2015). Free glucose was determined by the glucose oxidase-peroxidase reaction using a commercial kit (Spinreact, Girona, Spain). Briefly, 5 µL of sample were mixed with 300 µL of glucose oxidase/peroxidase reagent and incubated for 10 min at 37 °C. Finally, absorbance was measured at 505 nm using (BioTek Epoch 2 Microplate Spectrophotometer, Winooski, VT, USA). A glucose calibration curve was used for quantification (0–11 mM). Analyses were done in triplicate and results expressed in mM or g of glucose per 100 g of sample.

2.6.2. Dietary fiber

Total, insoluble and soluble dietary fibers, of biscuits were determined according to AOAC 991.43 (AOAC, 2012). FOS were not quantified by this method, so its content was added up according to product specification sheet. Analyses were performed at least in duplicate and results expressed as g in 100 g of sample.

2.6.3. Protein and amino acids

2.6.3.1. *Protein content*. Total protein content of biscuits was determined following AOAC 984.13 (AOAC, 2012). Analyses were performed in triplicate and results expressed as g in 100 g of sample.

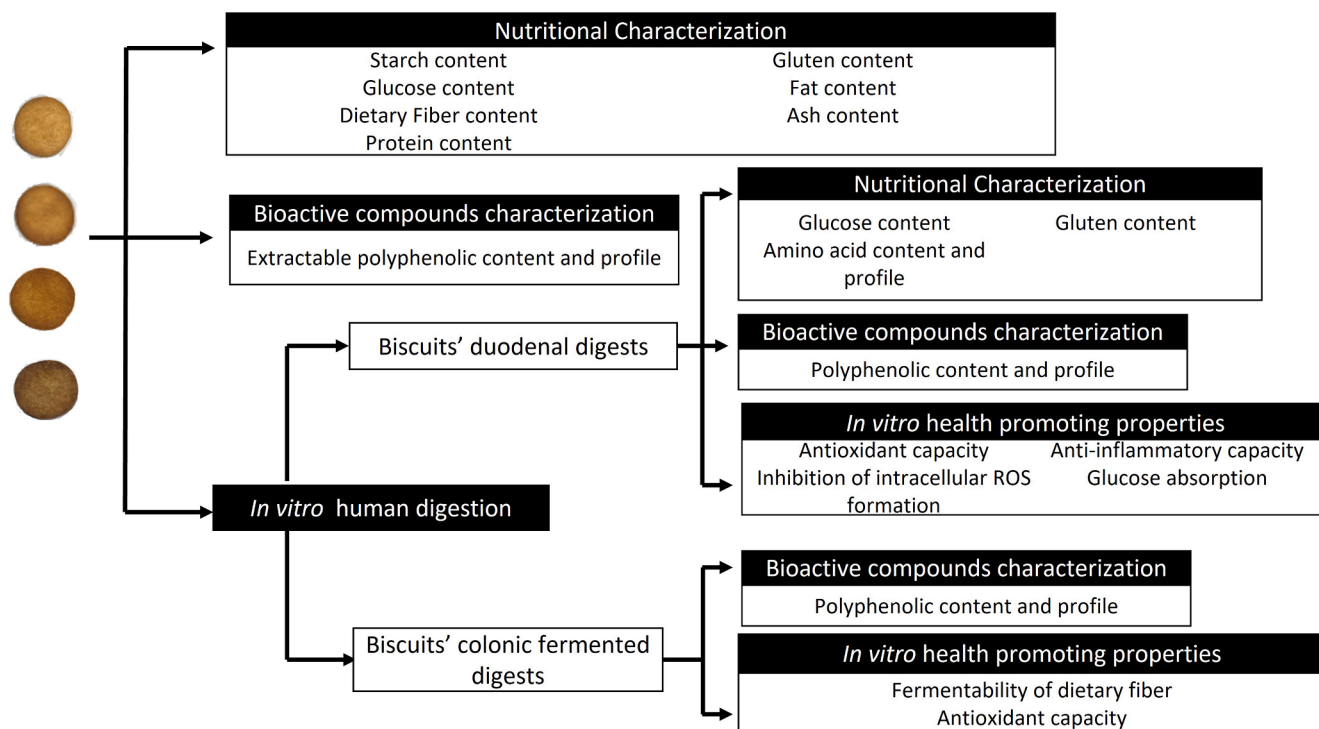


Fig. 2. Schematic summary of the methodology followed in the present study. ROS: Reactive oxygen species.

2.6.3.2. Gluten content. Gluten content of biscuits and their intestinal digests (D-TB, D-FOS-EBSG0, D-FOS-EBSG8, D-FOS-EBSG17) was determined by the enzyme-linked competitive immunoassay R5 method described by Mena et al (Mena, Lombardía, Hernando, Méndez, & Albar, 2012). Analyses were done in triplicate and results expressed in ppm.

2.6.3.3. Free amino acids. The total amino acids present in intestinal digests of biscuits (D-TB, D-FOS-EBSG0, D-FOS-EBSG8, D-FOS-EBSG17) was determined by o-phthalaldehyde (OPA) assay as described by Shene et al (Shene et al., 2020), adapted to micromethod. Five mg of OPA were dissolved in 100 μ L of ethanol (96 %), 5 μ L of β -2- mercaptoethanol and 10 mL of 50 mM carbonate buffer (pH 10.5). Fifty μ L of sample were mixed with 200 μ L of OPA reagent, in a quartz microplate. Finally, absorbance was measured during 1 min at 340 nm using a microplate reader (BioTek Epoch 2 Microplate Spectrophotometer, Winooski, VT, USA). Analyses were carried out in triplicate. N-acetyl-lysine (0–1.20 mM) curve was done for quantification. Results were expressed in mM of N-acetyl-lysine equivalents.

To determine the amino acid (AA) profile of intestinal digests of biscuits, 0.5 μ L of D-TB, D-FOS-EBSG0, D-FOS-EBSG8 and D-FOS-EBSG17 were analyzed using a Biochrom30 series amino acid analyzer (Biochrom Ltd., Cambridge Science Park, Cambridge, UK) according to Spackman et al (Spackman, Stein, & Moore, 1958) based on ion exchange chromatography and post column derivatization with ninhydrin. Determinations were done in triplicate and results were expressed in mM of AA.

2.6.4. Fat

Fat content of biscuits was determined following ISO-6492–1999. Analyses were performed in triplicate and results expressed as g in 100 g of sample.

2.6.5. Ashes

Ash content of biscuits was quantified based on ISO 5984–2002. Analyses were carried out in triplicate and results expressed as g in 100 g of sample.

2.6.6. Bioactive compounds (phenolic compounds)

2.6.6.1. Biscuits' phenolic compounds extraction. A hydro-alcoholic acid extraction (EHAA) of phenolic compounds in biscuits (TB, FOS-EBSG0, FOS-EBSG8, FOS-EBSG17) was performed according to Fernández et al (Fernández et al., 2020). Briefly, 1 g of each sample was mixed with 10 mL of extraction solvent (methanol:water:formic acid, 70:25:25). The tubes were kept under agitation (500 rpm) during 24 h at ambient temperature in the dark. Finally, they were centrifuged at 10.000 rpm and supernatants corresponded to hydro-alcoholic acid extracts. Extractions were made in triplicate for each biscuit.

2.6.6.2. Total phenolic content. Phenolic content was determined on extracts obtained in Section 2.6.6.1, intestinal digests of biscuits (D-TB, D-FOS-EBSG0, D-FOS-EBSG8 and D-FOS-EBSG17) and biscuits' colonic fermented digests (F-TB, F-FOS-EBSG0, F-FOS-EBSG8 and F-FOS-EBSG17). The method used was Folin-Ciocalteu method (Singleton & Rossi, 1965) adapted to micromethod as described in Iriondo-Dehond et al (Iriondo-Dehond et al. (2019)). Ferulic acid (FA) calibration curve was done for quantification (0.0–3.6 mM). Experiments were done in triplicate and results expressed as mg of ferulic acid equivalent (FAeq) per gram of sample.

2.6.6.3. Analysis of phenolic compounds by HPLC-QTOF assay. The identification of phenolic compounds by HPLC-QTOF was performed in extracts obtained in Section 2.6.6.1, intestinal digests of biscuits (D-TB, D-FOS-EBSG0, D-FOS-EBSG8 and D-FOS-EBSG17) and colonic fermented digests of biscuits (F-TB, F-FOS-EBSG0, F-FOS-EBSG8 and F-FOS-EBSG17). An HPLC equipment (Agilent 1200) with a quaternary pump (G1311A), coupled degasser (G1322A), thermostated automatic injector (G1367B), thermostated column module (G1316A) and diode array detector (G1315B), was used. It was coupled to a mass spectrometer (Agilent G6530A Accurate Mass QTOF LC/MS) with atmospheric pressure electrospray ionization source with JetStream technology. Control softwares were Masshunter Data Acquisition (B.05.00) and Masshunter Qualitative Analysis (B.07.00). Samples and

all standard solutions were injected at a volume of 20 μL in a ZORBAX Eclipse XDB-C18 column (150 mm \times 4.6 mm \times 5 μm) at 40 $^{\circ}\text{C}$. The solvent systems were 0.1 % formic acid (solvent A) and 0.1 % formic acid diluted in acetonitrile (solvent B). The elution gradient was (time, % of solvent A): 0 min, 95 %; 20 min, 85 %; 30 min, 70 %; 35 min, 50 %, 37 min, 95 %, 45 min, 90 %. A ferulic acid calibration curve was done (1–16 $\mu\text{g}/\text{mL}$). Identification was performed by comparison of the molecular formula, retention time, and previous references. Results are presented in [supplementary material](#).

2.7. In vitro health promoting properties assessment of biscuits

2.7.1. Antioxidant capacity

2.7.1.1. ABTS. Antioxidant capacity of biscuits intestinal digests (D-TB, D-FOS-EBSG0, D-FOS-EBSG8, D-FOS-EBSG17) and colonic fermented digests (F-TB, F-FOS-EBSG0, F-FOS-EBSG8, F-FOS-EBSG17) was measured by ABTS method proposed by Re et al (Re et al., 1999) and adapted to micro method as described by Martinez-Saez (Martinez-Saez et al., 2017). Ferulic acid (FA) calibration curve was used for quantification (0 – 80 μM). Experiments were done in triplicate and results expressed in mM FAeq.

2.7.1.2. ORAC. Total antioxidant capacity by oxygen radical absorbance capacity (ORAC) was performed according to Ou et al (Ou, Chang, Huang, & Prior, 2013) to intestinal and colonic fermented digests of biscuits. Twenty-five μL of samples were mixed with 150 μL of fluorescein ($11.12 \times 10^{-2} \mu\text{M}$) and incubated for 30 min at 37 $^{\circ}\text{C}$. Then, 25 μL of AAPH ($15.3 \times 10^{-2} \text{M}$) were included to start the reaction. Its kinetic was followed during an hour at 37 $^{\circ}\text{C}$ by measuring fluorescence every minute in a microplate reader (BioTek Cytation5 Cell Imaging Multi-Mode Reader, Winooski, VT, USA) at excitation and emission wavelengths of 485 nm and 530 nm, respectively. A ferulic acid calibration curve was done for quantification (0 – 30 μM). All measurements were performed in triplicate and results expressed as mM FAeq.

2.7.1.3. Intracellular reactive oxygen species' (ROS) formation. Intestinal digests of biscuits (D-TB, D-FOS-EBSG0, D-FOS-EBSG8, D-FOS-EBSG17) inhibition of intracellular reactive oxygen species (ROS) formation under physiological conditions in IEC-6 cells was determined using the fluorescent probe DCFH-DA, as described by Iriondo-Dehond (Iriondo-DeHond, Ramirez, Velazquez Escobar, & del Castillo, 2019). The digests' concentration to be applied in cells was determined previously by MTT assay (Bakondi et al., 2003) to guarantee at least 80 % of cell viability. Results are shown in [Supplementary Material \(Figure S2\)](#) and the digest concentration to apply in cells was 15 % v/v.

IEC-6 cells were seeded at a density of 2×10^4 cells/well on a 96-well plate and cultured in complete medium (DMEM with 4.5 g/L of glucose, 10 % v/v of FBS, 1 %v/v of L-glutamine and 1 % v/v of antibiotics) for 24 h (37 $^{\circ}\text{C}$, 5 % CO_2). Afterwards, medium was aspirated, and cells were loaded with 100 μL containing biscuits' intestinal digests (15 % v/v) and medium without FBS at a ratio equal to 1:10. After 24 h incubation period, wells' content was aspirated, and cells were pre-loaded with 100 μL of medium without FBS containing 2 μL of DCFH-DA (0.3 mg/mL in DMSO). Cells were incubated for 45 min. Then, wells' content was aspirated, cells were washed with PBS and were treated with 100 μL containing biscuits' intestinal digests (15 % v/v) and medium without FBS at a ratio equal to 1:10 for 30 min. Tert-butylhydroperoxide (tBOOH) 1 mM was used as an oxidation control and ascorbic acid (10 $\mu\text{g}/\text{mL}$) was used as antioxidant control. A control was done by adding only medium without FBS to establish ROS formation at assay conditions. Afterwards, fluorescence was measured at 485 nm/528 nm (BioTek Synergy HT Multi-Mode Microplate Reader). Finally, 20 μL of MTT reagent were added per well, plates were incubated for 90 min. Wells' content was removed, 100 μL of DMSO were added to each well

and absorbance measured at 570 nm using a BioTek Epoch 2 Microplate Spectrophotometer (Winooski, VT, USA) (Bakondi et al., 2003). All measurements were done in triplicate and in three different cell passages. Intracellular ROS formation was calculated as follows:

$$\%ROS \text{ formation} = \frac{\text{Fluorescence}_{\text{Sample}} / \text{MTT Absorbance}_{\text{Sample}}}{\text{Fluorescence}_{\text{Control}} / \text{MTT Absorbance}_{\text{Control}}} \times 100 \quad (1)$$

2.7.2. Anti-inflammatory effect

Anti-inflammatory properties of intestinal digests of biscuits (D-TB, D-FOS-EBSG0, D-FOS-EBSG8, D-FOS-EBSG17) were determined by quantifying the nitrogen oxide (NO) production in macrophages (RAW264.7) as described by Benayad et al (Benayad, Martinez-Villaluenga, Frias, Gomez-Cordoves, & Es-Safi, 2014). The concentration to be used of biscuits' intestinal digests was determined by MTT assay (Bakondi et al., 2003), prior to analysis, and results are shown in [Supplementary Material \(Figure S3\)](#). The intestinal digest of biscuits concentration applied to cells was 15 % v/v. Negative and positive controls were tested consisting of medium without FBS and 1 $\mu\text{g}/\text{mL}$ of LPS in medium without FBS, respectively. A NO in DMEM without FBS calibration curve was done for quantification (0–10 $\mu\text{g}/\text{mL}$). Analyses were done in triplicate and for three different cell passages.

2.7.3. Glucose absorption: Antidiabetic properties

Glucose absorption in IEC-6 was determined for D-FOS-EBSG0, D-FOS-EBSG8 and D-FOS-EBSG17. A control containing the maximum glucose concentration in the biscuits' intestinal digests was done. Transwell plates with polycarbonate inserts (Transwell® inserts, 0.4 μm pore size, 1.1 cm^2) were used. Cells were seeded on 12 well Transwell plates with 7.6×10^4 cells/well. Cells were cultivated (37 $^{\circ}\text{C}$, 5 % CO_2) for 10 days, to differentiate. Five hundred μL and 1500 μL of medium (DMEM with 4.5 g/L of glucose, 10 % v/v of FBS, 1 %v/v of L-glutamine and 1 % v/v of antibiotics) were added on the apical and basolateral sides of each well, respectively. Medium was changed every 48 h. Transepithelial electrical resistance (TEER) was measured using a Millicell-ERS device (Millipore, Zug, Switzerland) to evaluate monolayers integrity.

The assay day, cells were washed with PBS 10 mM and pH 7.4. Then, PBS was aspirated, and cells incubated (37 $^{\circ}\text{C}$, 5 % CO_2) for 30 min with PBS in absence of glucose. After the incubation period, PBS was removed from both sides of the Transwell plate and replaced by 500 μL of tested sample prepared in PBS in the apical side and 1500 μL of PBS in the basolateral side. Cells were incubated (37 $^{\circ}\text{C}$, 5 % CO_2) and 200 μL were removed from the basolateral side at different times (10 min, 30 min, 45 min, 60 min, 75 min, 90 min and 120 min) and volume in the basolateral side was replaced with PBS.

Lucifer yellow test was done after the assay had finished (Jennis et al., 2017; Miller, Monsul, Vender, & Lehmann, 1996; Puthia, Sio, Lu, & Tan, 2006). Wells were washed with PBS and 200 μL of lucifer yellow reagent (50 μM) were put in the apical side. One thousand five hundred μL of PBS were added in the basolateral side and plates were incubated for 1 h at 37 $^{\circ}\text{C}$. Finally, 200 μL aliquots from the basolateral side were removed and its fluorescence was measured (485 nm/528 nm). Permeability percentage was calculated as the coefficient between samples fluorescence and lucifer yellow reagent (50 μM) fluorescence. Permeability <5 % was considered adequate.

Glucose absorption was determined by measuring glucose concentration (see Section 2.6.1.3) in the basolateral side at different times by duplicate. Glucose contents at different times were plotted in a graph and area under curve (AUC) was calculated. Statistical analysis was made between the AUCs determined for each biscuit intestinal digest between 10 min and 120 min.

2.7.4. Bioconversion of biscuits dietary fiber by gut microbiota

2.7.4.1. Organic acids. Organic acid analysis was made to quantify

lactic acid and succinic acid in colonic fermented digests of biscuits (F-TB, F-FOS-EBSG0, F-FOS-EBSG8 and F-FOS-EBSG17). Metrohm Advanced Compact ion chromatography instrument (867 IC, Metrohm) equipped with a conductivity detector (IC-819), 889 IC Sample Center injector, and coupled with degasser (IC-837) was used. Samples, lactate standard (07096-100ML, Sigma-Aldrich) and succinate standard (43057-100 ML, Sigma Aldrich) were injected at a volume of 20 μ L in a Metrosep Organic Acids column (250 \times 4 mm, 5 μ m). The mobile phase was 0.5 mM sulfuric acid and 15 % acetone, 0.5 mL/minute for 20 min. Identification was performed by comparison of retention time with patterns used. Control software were Metrodata IC Net 2.3 and MagiIC Net 2.3 (Metrohm, Switzerland). Analyses were done in triplicate.

2.7.4.2. Sugars. Identification and quantification of sugars (glucose, arabinose, xylose, fructose) in colonic fermented digests of biscuits (F-TB, F-FOS-EBSG0, F-FOS-EBSG8 and F-FOS-EBSG17) was done by ion exchange liquid chromatography (LC-IC). Standards used for quantification were D (+) glucose (1.08337.0250, Merck), D (-) arabinose and D (-) Fructose (1.04007.0250, Merck). Equipment used consisted of Metrohm Advanced Compact ion chromatographic instrument with Bioscan module (817 IC, Metrohm, Switzerland), equipped with a Pulse Amperometric Detector (PAD) (945 Professional Detector Vario), a pump (IC Pump 812), 889 IC Sample Center injector, and coupled with a degasser (IC-837). Two to 100 μ L of samples and standards were injected in Metrosep Carb 2 column (250 \times 4 mm). The mobile phase used was 300 mM sodium hydroxide and 1 mM sodium acetate, applied at a flow rate of 0.5 mL/minute for 35 min. Identification was performed by comparison of retention time with patterns used. Data were analyzed using Metrodata IC Net 2.3 and MagiIC Net 2.3 (Metrohm, Switzerland). Analyses were performed in triplicate.

2.7.4.3. Short-chain fatty acids. Short-chain fatty acids (SCFAs) were determined in colonic fermented digests of biscuits (F-TB, F-FOS-EBSG0, F-FOS-EBSG8, F-FOS-EBSG17) and in fecal inoculum, to establish the baseline. Samples or standards (400 μ L) were acidified with 200 μ L of phosphoric acid (0.5 %). One hundred μ L of methyl valeric (8092 μ M) were added as internal standard and were extracted with 1000 μ L of n-butanol. Identification and quantification of SCFAs was done using a gas chromatography (Agilent 6890A) equipped with a flame ionization detector (260 $^{\circ}$ C), an automatic injector (G2613A) and a DB-WAXtr column (60 m \times 0.325 mm \times 0.25 μ m) (Agilent Technologies). Two μ L of samples or standards were injected splitless at 250 $^{\circ}$ C. The carrier gas used was helium at a constant flow rate (1.5 mL/min). The initial column temperature was 50 $^{\circ}$ C and held 2 min, increased to 150 $^{\circ}$ C at the rate of 15 $^{\circ}$ C/min, to 200 $^{\circ}$ C at 5 $^{\circ}$ C/min and increased to 240 $^{\circ}$ C at the rate of 15 $^{\circ}$ C/min and kept for 20 min. The control software used was MSD Chemstation E.02.00.493. Analyses were done in triplicate and results were expressed in mM.

2.8. Statistical data analysis

All results were reported as mean \pm standard deviation. One way analysis of variance was performed on each assay, and differences between samples were determined by the Tukey test ($\alpha \leq 0.05$). Analyses were done with XLSTAT Version 2011(Addinsoft 1995–2010, France). Pearson correlation was used with significance level of 0.05.

3. Results and discussion

3.1. Nutritional characterization of biscuits

Table 1 shows tested biscuits recipe. Traditional biscuit recipe was made based on “Marie” biscuit. This semi-sweet biscuit recipe consists of granulated sugar (15–18 %) as the main sweetener and semi-solid fat (9–15 %) addition as a critical ingredient (Sykes & Davidson, 2020).

Formulations of healthier snacks, with high organoleptic and nutritional qualities were proposed. Butter content was reduced by 30 % compared to traditional added sugar biscuit to preserve the palatability of the original product. Fat contributes to texture, flavor and aroma in a wide variety of foods, while the first sensory response tends to be the olfactory perception of fat-soluble volatile flavor molecules (Drewnowski & Almiron-Roig, 2010). Furthermore, 70 % of sucrose content was reduced and substituted by FOS which is an undigestible carbohydrate with 30 %–50 % of sweetening power and positive effects on gut and cognitive health (Silva, Bernardi, & Frozza, 2020) for satisfying actual consumer demands. Furthermore, levels of added sugar, shown in Table 1, were equal to 4.9 %. In the same line, extruded brewers’ spent grain (EBSG) was used to reduce refine wheat flour content and improve products sustainability. EBSG is a source of insoluble dietary fibre (46.70 % \pm 0.63 %), protein (29.46 % \pm 0.11 %) and bioactive compounds (Gutiérrez-Barrutia et al., 2022), which may contribute to biscuits’ potential health claims.

Table 2 shows the biscuits’ proximate composition. As can be observed, all reduced sugar biscuits had >6 % of dietary fiber in their composition. Following FOS product specification sheet: 89 % of FOS (Orafti® P95) and 69 % of FOS (Orafti® L95) content corresponded to low molecular weight dietary fiber which is not quantified by enzymatic-gravimetric method. Thus, from 10.2 % of FOS (Orafti® P95) and 5 % of FOS (Orafti® L95) added to each reduced sugar biscuit (Table 1), a total of 12.53 % corresponded to soluble dietary fiber. In line with previous studies (Farcas et al., 2021; Ktenioudaki et al., 2013; Öztürk,

Table 2
Biscuits’ proximate composition.

	TB	FOS-EBSG0	FOS-EBSG8	FOS-EBSG17
TDF*(g/100 g)	2.035 \pm 0.001 ^{a*}	14.755 \pm 0.054 ^{b*}	17.698 \pm 0.141 ^{c*}	20.852 \pm 0.125 ^{d*}
IDF (g/100 g)	1.450 \pm 0.059 ^a	1.543 \pm 0.045 ^a	4.171 \pm 0.195 ^b	7.222 \pm 0.149 ^c
SDF (g/100 g)	0.586 \pm 0.039 ^a	0.682 \pm 0.008 ^a	0.997 \pm 0.022 ^b	1.100 \pm 0.025 ^c
Total starch (g/100 g)	44.667 \pm 2.215 ^c	45.921 \pm 1.594 ^c	39.023 \pm 1.555 ^b	31.693 \pm 0.654 ^a
Digestible starch (g/100 g)	43.585 \pm 1.832 ^c	45.178 \pm 1.806 ^c	38.336 \pm 1.256 ^b	30.970 \pm 0.910 ^a
Resistant starch (g/100 g)	0.245 \pm 0.038 ^a	0.252 \pm 0.013 ^a	0.652 \pm 0.050 ^c	0.567 \pm 0.012 ^b
Free glucose (g/100 g)	0.039 \pm 0.004 ^a	0.091 \pm 0.004 ^b	0.127 \pm 0.005 ^c	0.131 \pm 0.008 ^c
Proteins (g/100 g)	8.228 \pm 0.142 ^a	8.672 \pm 0.116 ^b	10.074 \pm 0.193 ^c	12.425 \pm 0.108 ^d
Gluten ($\times 10^4$ ppm)	3.967 \pm 0.548 ^b	3.066 \pm 0.625 ^a	5.411 \pm 0.557 ^c	5.143 \pm 1.705 ^c
Fat (g/100 g)	16.114 \pm 0.149 ^d	11.313 \pm 0.002 ^a	11.674 \pm 0.147 ^b	12.926 \pm 0.048 ^c
Ash (g/100 g)	0.889 \pm 0.043	0.882 \pm 0.022	1.312 \pm 0.050	1.534 \pm 0.003
Calories** (kcal/100 g)	455.2	401.8	395.6	396.8

Results shown as mean \pm standard deviation. Different letters within the same line show significant differences ($p < 0.05$). TB: Traditional biscuit. FOS-EBSG0: Reduced sugar biscuit with no extruded brewers’ spent grain. FOS-EBSG8: Reduced sugar biscuit with 8 % extruded brewers’ spent grain. FOS-EBSG17: Reduced sugar biscuit with 17 % extruded brewers’ spent grain. SDF: Soluble dietary fiber. IDF: Insoluble dietary fiber. TDF: Total dietary fiber.

* TDF was calculated as IDF + SDF + added FOS dietary fiber content which corresponded to 89 % of FOS (Orafti® P95) and 69 % of (FOS Orafti® L95). Added FOS dietary fiber was 12.53 %.

** Calories were calculated by summing up the energy values of nutrients composing biscuits (4 kcal/g of protein and carbohydrate, 9 kcal/g of fat, 2 kcal/g of dietary fibre), for this calculation total carbohydrate content was calculated by difference. Biscuit moisture content was: 4.342 \pm 0.028, 5.444 \pm 0.220, 5.530 \pm 0.032 and 5.010 \pm 0.027 g/100 g for TB, FOS-EBSG0, FOS-EBSG8 and FOS-EBSG17, respectively.

Özboy, Ox, Köksel, & Brew, 2002; Petrović et al., 2015), as expected, substitution of wheat flour with EBSG resulted in a significant increase ($p < 0.05$) of the biscuits' dietary fiber content. Previous formulated biscuits with 15 % of fine dried BSG had 8.23 % of dietary fiber (Öztürk et al., 2002). Formulated biscuits containing 15 %, 25 % and 50 % of fresh BSG, presented a total dietary fiber equal to 6.80 ± 0.47 , 8.50 ± 0.45 and 15.55 ± 0.78 g/100 g, respectively (Petrović et al., 2015). The crude fiber of biscuits' containing 9.46 g/100 g BSG ranged from 3.22 ± 0.11 and 3.38 ± 0.12 g/100 g (Farcas et al., 2021).

Furthermore, a significant ($p < 0.05$) reduction of digestible starch content was observed in biscuits as the level of EBSG increased. The content of digestible starch of EBSG was 2.55 ± 0.06 g/100 g (Gutiérrez-Barrutia et al., 2022), which is lower than the one for refined wheat flour. Biscuit with 17 % of EBSG resulted in a decrease by 1.5 folds in its total starch compared to biscuits without EBSG. Similarly, a reduction by 1.14 and 1.29 folds in total starch content of breadsticks was achieved when wheat flour was substituted with 15 % and 25 % of BSG, respectively (Ktenioudaki et al., 2013). Additionally, biscuits with EBSG had significantly ($p < 0.05$) higher content of resistant starch than biscuits without it. Similar trends were found for pasta formulated with 10 % or 20 % of BSG instead of semolina (Nocente, Taddei, Galassi, & Gazza, 2019). Physiological benefits associated to resistant starch containing food intake were mainly linked to resistant starch fermentation in the large intestine by the gut microbiota (Bojarczuk, Skapska, Mousavi Khaneghah, & Marszałek, 2022).

The amount of free glucose of the biscuits is low (Table 2) and comes mainly from wheat flour, FOS and EBSG. Interestingly, traditional biscuit was the one with lower amount of free glucose ($p < 0.05$). This could be expected as its main sweetener is sucrose which contains glucose bounded to fructose and will not contribute to free forms of glucose unless hydrolyzed. Therefore, the amount of free glucose in traditional biscuits may come principally from wheat flour ingredient, whose sugar content has been reported equal to 0.27 % (USDA, 2019). Additionally, according to product specification sheet, 8 % of FOS (Orafti® P95) and 6 % of FOS (Orafti® L95) content corresponds to free sugars, resulting from the endoinulinase activity used for their obtention (Singh, Singh, & Kennedy, 2016). Thus, the addition of 10.2 % of FOS (Orafti® P95) and 5.0 % of FOS (Orafti® L95) results in 1.1 % of free sugars added to reduced sugar biscuits. Besides, EBSG has 0.54 ± 0.02 % of free glucose (Gutiérrez-Barrutia et al., 2022). Therefore, both FOS and EBSG contributed to the higher level of free glucose detected in FOS-EBSG0, FOS-EBSG8 and FOS-EBSG17. Nevertheless, this may not result in a higher glycemic index for these biscuits compared to TB. Glycemic index depends on the digestible carbohydrates composing the food resulting in bioaccessible glucose that can be absorbed in the enterocytes into blood stream, after human oral-gastro-intestinal digestion.

Based on results presented in Table 2, wheat flour substitution with EBSG increased ($p < 0.05$) biscuits' protein content, as found in previous studies (Farcas et al., 2021; Heredia-Sandoval et al., 2019; Ktenioudaki et al., 2013; Petrović et al., 2015). Besides, 12 % of the energy value for biscuits with 17 % EBSG is given by their proteins (Table 2). The addition of 8 % and 17 % EBSG resulted in an increase of the biscuits protein content by 1.15 and 1.45 folds, respectively, compared to reduced sugar biscuit without EBSG. An increase in biscuits' protein content by 1.33 has been reported when 20 % of wheat flour is substituted by dry BSG (Heredia-Sandoval et al., 2019). Different protein contents have been found for biscuits' containing BSG, which ranged from 7.55 ± 0.41 % for biscuits containing 15 % fresh BSG (Petrović et al., 2015) to 8.89 ± 0.03 % for biscuits containing 9.46 % of dry BSG (Farcas et al., 2021).

EBSG biscuits had significantly ($p < 0.05$) more gluten than biscuits without EBSG, what may be due to the higher content of gluten in EBSG than in wheat flour. It has been reported that EBSG contains $(25.258 \pm 1.413) \times 10^5$ ppm of gluten (Gutiérrez-Barrutia et al., 2022), while soft wheat flour gluten content ranged from $(2.30-4.73) \times 10^4$ ppm (Yu et al., 2021).

Similarly, the addition of EBSG caused an increase in fat content ($p < 0.05$). EBSG has 10.34 ± 0.01 g/100 g of lipids in dry weight basis (Gutiérrez-Barrutia et al., 2022), while wheat flour lipid content is 0.98 g/100 g (USDA, 2019). Nevertheless, the addition of BSG in biscuits may contribute to an improved lipid profile as almost half of BSG fatty acids correspond to linoleic and oleic acid (Niemi et al., 2012).

3.2. Bioactive compounds of biscuits

Extractable phenolic content (EPC) of the biscuits measured by Folin-Ciocalteu is shown in Table 3. EBSG addition resulted in a higher EPC ($p < 0.05$). Same trend was found for pasta formulated with fermented and native BSG compared to wheat pasta (Schettino et al., 2021). Furthermore, the addition of 9.5 g /100 g of BSG in biscuits generated the EPC level at a range of 47–55 mg GAE /100 g (Farcas et al., 2021), which is aligned with results obtained for biscuits with 8 g/100 g of EBSG in the current study (53.0 ± 0.4 mgGAE/100 g). The identification of phenolic compounds by HPLC-QTOF in hydroalcoholic extracts of biscuits is shown in Supplementary material (Table S2, Figure S1).

3.3. Intestinal digests of biscuits

3.3.1. Nutritional characterization

Results for bioaccessible nutrients after *in vitro* oral-gastro-intestinal digestion are presented in Table 4. Intestinal bioaccessible glucose level of traditional biscuit and reduced sugar biscuit without EBSG was statistically the same ($p > 0.05$). Thus, no reduction in bioaccessible glucose levels were seen by the substitution of sucrose by FOS. However, the substitution of wheat flour by EBSG in biscuits, significantly ($p < 0.05$) reduced the glucose bioaccessibility. In fact, EBSG biscuits glucose bioaccessibility levels is near the limits of what is considered normal glucose levels in humans' blood under fasting conditions (4.4 – 6.1 mM) (Gromova, Fetissov, & Gruzdkov, 2021). This may be due to the lower ($p < 0.05$) digestible starch level of EBSG biscuits compared to biscuits without EBSG (Table 2). In human digestive process, digestible starch is met with salivary amylase in the mouth, pancreatic fluids and *exo*-glucosidases which are integral to the plasma membrane of enterocytes in the small intestine, which altogether yield the release of mono-saccharides (glucose) (Dona, Pages, Gilbert, & Kuchel, 2010). Thus, results showed biscuits' glucose bioaccessibility was mostly conditioned by starch content than by free glucose or sucrose content, as EBSG-added biscuits presented a higher amount of free glucose than biscuits without EBSG (Table 2). A previous study involving 12 healthy volunteers, reported a reduction in glycemic index by substituting starch with sucrose in high glycemic index cereals. Currently highly digestible starches are considered to be more damaging than sucrose, which is less glycemic. The majority of starch in food products result in a rapid release of

Table 3

Extractable and bioaccessible phenolic compounds in biscuits.

	Total polyphenolic content (mg Faeq/ g of sample)		
	EHAA extracts	Duodenal Bioaccessibility	Colonic Bioaccessibility
TB	0.140 ± 0.001^a	1.588 ± 0.078^a	5.236 ± 0.156^b
FOS-EBSG0	0.336 ± 0.004^b	1.901 ± 0.097^b	4.924 ± 0.169^a
FOS-EBSG8	0.414 ± 0.008^c	2.091 ± 0.066^c	5.249 ± 0.224^b
FOS-EBSG17	0.941 ± 0.049^d	2.480 ± 0.141^d	4.637 ± 0.145^a

Different letters within the same column show significant differences ($p < 0.05$). EHAA: Hydroalcoholic-acid extraction. TB: Traditional biscuit. FOS-EBSG0: Reduced sugar biscuit with no extruded brewers' spent grain. FOS-EBSG8: Reduced sugar biscuit with 8 % extruded brewers' spent grain. FOS-EBSG17: Reduced sugar biscuit with 17 % extruded brewers' spent grain.

Table 4
Nutrients' bioaccessibility in biscuits' duodenal digests.

	Glucose (mM)	Gluten (ppm)	Amino acids (mM of equivalent N-acetyl lysine)
TB	13.703 ± 0.668 ^b	<3 ^a	33.186 ± 1.756 ^a
D-FOS-EBSG0	13.890 ± 0.919 ^b	<3 ^a	31.635 ± 3.013 ^a
D-FOS-EBSG8	6.180 ± 0.336 ^a	<3 ^a	35.788 ± 1.583 ^a
D-FOS-EBSG17	6.389 ± 0.288 ^a	<3 ^a	36.174 ± 2.108 ^a

Different letters within the same column show significant differences ($p < 0.05$). D-TB: Tradition biscuit intestinal digest. D-FOS-EBSG0: Reduced sugar biscuit without extruded brewers' spent grain intestinal digest. D-FOS-EBSG8: Reduced sugar biscuit with 8 % extruded brewers' spent grain intestinal digest. D-FOS-EBSG17: Reduced sugar biscuit with 17 % extruded brewers' spent grain intestinal digest.

glucose and so promote substantial glycaemia (Brand-Miller & Lobbezoo, 1994).

Despite the higher protein content in EBSG-added biscuits compared to biscuits without EBSG, no significant differences ($p > 0.05$) were found in the total content of free amino acids released during oral-gastro-intestinal digestion of biscuits, as shown in Table 4 and Table 5. Thus, EBSG addition to biscuits allowed to keep the levels of bioaccessible amino acids of the original biscuit recipe although levels of wheat flour were reduced. The higher ($p < 0.05$) content of dietary fiber in EBSG biscuits might have hindered protein digestibility. It was found that diets with whole oat as protein source caused a reduction in the percentage of true nitrogen digestibility in rats. Moreover, rats fed with cellulose presented a decrease in the protein efficiency ratio when cellulose levels were beyond 5 % (Barrón Hoyos, Domínguez Salazar, & Falcón Villa, 2013). Amino acids which are not bioaccessible at the duodenum may be later metabolize by gut microbiota to form branched short chain fatty acids as discussed in section 3.4.2.2. Nevertheless, gluten was not detected in any of the biscuit's digested fractions. Therefore, gluten digestibility was not affected, beyond possible dietary fiber interaction in protein digestibility.

Regarding essential amino acids (EAA) (Table 5), lysine bioaccessibility was significantly ($p < 0.05$) lower in biscuits containing 17 % EBSG than in traditional sweet biscuit. Results for biscuits' intestinal digests advanced glycation end products (AGEs) are shown in Supplementary Material (Table S1). Favored by the slight alkaline at duodenal digestion stage, reaction between amino acids and reducing sugars may result in advanced glycation end (AGEs) products (Martinez-Saez, Fernandez-Gomez, Cai, Uribarri, & del Castillo, 2019), what may reduce amino acids bioaccessibility. Lysine was shown to be the most reactive amino acid in Maillard model systems and the key contributor to Maillard reaction products produced through protein glycation (Hemm et al., 2018). In fact, a negative Pearson correlation ($p < 0.05$, -0.72) was found between the lysine content of biscuit's intestinal digests and their AGEs' content.

Focusing on the amino acid profile of biscuits' intestinal digests (Table 5), leucine was the major amino acid registered followed by valine, glycine and alanine. The main sources of amino acids in biscuits were eggs and EBSG. Previous works have established that glutamic acid, leucine and aspartic acid were the major amino acids in eggs (Iriando-Dehond, Salazar, Guspí Domènech, Zapata Montoya, & del Castillo, 2022), while leucine, glutamic acid and proline were in EBSG (Gutiérrez-Barrutia et al., 2022). Besides, leucine (8.6 %) and valine (5.4 %) were the major EAA registered in biscuits formulated with 15 % BSG (Kissell & Prentice, 1979). Milligrams of leucine released during biscuits' oral-gastro-intestinal digestion were 7.232 ± 0.444 , 7.916 ± 0.864 , 7.389 ± 0.108 and 6.913 ± 0.246 for TB, FOS-EBSG0, FOS-EBSG8 and FOS-EBSG17 respectively. These values represent between

Table 5
Amino acid profile for biscuits intestinal digests.

	Amino acid (mM)	D-TB	D-FOS-EBSG0	D-FOS-EBSG8	D-FOS-EBSG17	
Nonessential amino acids (NEAA)	Alanine	3.130 ± 0.375 ^{a,F,G,H}	2.822 ± 0.597 ^{a,E,F,G}	2.942 ± 0.088 ^{a,G}	3.231 ± 0.072 ^{a,G,H}	
	Arginine	3.207 ± 0.387 ^{a,F,G,H}	2.903 ± 0.571 ^{a,E,F,G}	2.785 ± 0.053 ^{a,G}	2.5133 ± 0.038 ^{a,F}	
	Aspartic acid	0.334 ± 0.008 ^{a,A}	0.139 ± 0.072 ^{a,A}	0.248 ± 0.006 ^{a,A}	0.186 ± 0.011 ^{a,A}	
	Cysteine	0.512 ± 0.090 ^{a,A,B}	0.525 ± 0.087 ^{a,A,B}	0.447 ± 0.018 ^{a,A,B}	0.377 ± 0.0351 ^{a,A,B}	
	Glutamic acid	1.185 ± 0.225 ^{a,B,C}	1.126 ± 0.217 ^{a,A,B,C}	0.978 ± 0.028 ^{a,C}	0.850 ± 0.040 ^{a,C}	
	Glycine	3.477 ± 0.215 ^{b,G,H}	3.281 ± 0.259 ^{a,b,F,G}	3.030 ± 0.032 ^{a,b,G,H}	2.940 ± 0.077 ^{a,G}	
	Proline	0.754 ± 0.140 ^{a,A,B,C}	0.543 ± 0.051 ^{a,A,B}	0.577 ± 0.152 ^{a,B}	0.580 ± 0.212 ^{a,B}	
	Serine	3.473 ± 0.474 ^{a,G,H}	3.272 ± 0.420 ^{a,F,G}	3.255 ± 0.156 ^{a,H}	2.995 ± 0.158 ^{a,G}	
	Tyrosine	1.507 ± 0.159 ^{a,C,D}	1.489 ± 0.203 ^{a,B,C,D}	1.444 ± 0.042 ^{a,E}	1.360 ± 0.028 ^{a,D}	
	Total NEAA	13.266 ± 1.966 ^a	12.112 ± 1.362 ^a	10.518 ± 0.418 ^a	10.015 ± 0.385 ^a	
	Essential amino acids (EAA)	Histidine	1.132 ± 0.099 ^{a,A,B,C}	1.111 ± 0.194 ^{a,A,B,C}	1.031 ± 0.029 ^{a,C,D}	0.938 ± 0.018 ^{a,C}
		Isoleucine	2.080 ± 0.250 ^{a,D,E}	2.125 ± 0.288 ^{a,C,D,E}	2.063 ± 0.014 ^{a,F}	1.872 ± 0.020 ^{a,E}
		Leucine	4.704 ± 0.531 ^{a,I}	4.66 ± 0.623 ^{a,b,H}	5.362 ± 0.071 ^{a,J}	5.279 ± 0.080 ^{a,I}
Lysine		2.657 ± 0.271 ^{b,E,F}	2.101 ± 0.453 ^{a,b,D,E,F}	2.013 ± 0.038 ^{a,b,F}	1.735 ± 0.037 ^{a,E}	
Methionine		1.219 ± 0.144 ^{a,B,C}	1.255 ± 0.233 ^{a,A,B,C,D}	1.233 ± 0.035 ^{a,D,E}	1.141 ± 0.028 ^{a,C,D}	
Phenylalanine		2.753 ± 0.225 ^{a,E,F,G}	3.022 ± 0.421 ^{a,E,F,G}	2.823 ± 0.063 ^{a,G}	2.571 ± 0.244 ^{a,F}	
Threonine		1.135 ± 0.151 ^{a,A,B,C}	1.004 ± 0.150 ^{a,A,B}	1.050 ± 0.021 ^{a,C,D}	0.909 ± 0.037 ^{a,C}	
Tryptophan		n.d	n.d	n.d	n.d	
Valine	3.708 ± 0.275 ^{a,H}	3.517 ± 0.598 ^{a,G}	3.545 ± 0.184 ^{a,I}	3.461 ± 0.027 ^{a,H}		
Total EAA	20.560 ± 0.260 ^a	19.110 ± 2.440 ^a	19.104 ± 0.271 ^a	18.037 ± 0.055 ^a		
Total	37.190 ± 3.950 ^a	35.341 ± 5.549 ^a	34.754 ± 0.700 ^a	32.800 ± 0.505 ^a		

Different letters within the same line (lower case) and column (capital letters) show significant differences ($p < 0.05$). D-TB: Tradition biscuit intestinal digest. D-FOS-EBSG0: Reduced sugar biscuit without extruded brewers' spent grain intestinal digest. D-FOS-EBSG8: Reduced sugar biscuit with 8 % extruded brewers' spent grain intestinal digest. D-FOS-EBSG17: Reduced sugar biscuit with 17 % extruded brewers' spent grain intestinal digest. n.d means not detected.

18 % and 19 % of leucine daily mean requirement for adults per kilogram of body weight (EFSA, 2012). In the case of valine, 5.678 ± 0.326 , 5.874 ± 0.673 , 5.358 ± 0.263 and 4.873 ± 0.102 mg were released from TB, FOS-EBSG0, FOS-EBSG8 and FOS-EBSG17 digestion, respectively, while 26 mg is the mean daily intake for adults per kilogram of body weight (EFSA, 2012). Branched amino acids (leucine, isoleucine, valine) intake was associated with a positively skeletal muscle mass index (Chae, Park, & Park, 2020). Moreover, high intakes of total branched chain amino acids were linked to a decreased risk of diabetes in women (Nagata et al., 2013).

3.3.2. Phenolic compounds characterization

Results for intestinal bioaccessible content of phenolic compounds of biscuits are shown in Table 3. The addition of EBSG generated significant ($p < 0.05$) higher levels of phenolic compounds duodenal bioaccessibility. Data suggested that the addition of EBSG provided phenolic compounds with potential health promoting properties. Similar results were found for bread made with BSG, with a maximum phenolic content in bread with 100 % non-squeezed BSG (746 mg GAE/100 g) and lowest content in the 10 % non-squeezed BSG bread (299 mg GAE/100 g) after digestion (Merten et al., 2022). These trends agreed with results obtained from the phenolic compounds identified by HPLC-QTOF assay. Results for this analysis are shown in supplementary material (Figure S1, Table S3).

3.3.3. In vitro health promoting properties of biscuits' intestinal digests.

3.3.3.1. Antioxidant properties. The antioxidant potential of the biscuits digested under intestinal conditions and colonic fermented are shown in Table 6.

Biscuits containing EBSG intestinal digests presented a significantly higher ($p < 0.05$) overall antioxidant capacity at the duodenal stage measured by ABTS and by ORAC methods compared to biscuits without EBSG. Data agreed with results presented for their phenolic compounds (Table 3). A significant ($p < 0.001$) and positive Pearson correlation was found between the phenolic content measured by Folin-Ciocalteu and the antioxidant capacity of biscuits' intestinal digests measured by ABTS ($r = 0.66$) and ORAC ($r = 0.99$). Similar trends were found previously by others. Pasta and bread formulated with BSG presented a higher antioxidant capacity after *in vitro* oral-gastro-intestinal digestion than their respective controls (Merten et al., 2022; Schettino et al., 2021).

To assess the bioactivity of biscuits' intestinal digests, their inhibition capacity on the formation of intracellular ROS in IEC-6 cells was studied (Fig. 3). All the biscuits' intestinal digests significantly ($p < 0.05$) reduced the levels of intracellular ROS formed. However, biscuits with 17 % EBSG presented the highest inhibition capacity which was statistically the same as the one for ascorbic acid (10 $\mu\text{g}/\text{mL}$). Thus, a

Table 6

Antioxidant capacity measured by ABTS and ORAC for biscuits' digested and fermented extracts.

	ABTS ($\mu\text{mol Faeq}/\text{g}$ of sample)		ORAC ($\mu\text{mol Faeq}/\text{g}$ of sample)	
	Duodenal	Colonic	Duodenal	Colonic
TB	18.536 \pm 0.264 ^a	33.920 \pm 1.574 ^b	7.871 \pm 0.109 ^a	17.194 \pm 0.147 ^b
FOS-EBSG0	20.432 \pm 1.000 ^b	30.836 \pm 1.717 ^b	8.036 \pm 0.009 ^a	17.885 \pm 0.139 ^b
FOS-EBSG8	22.413 \pm 0.668 ^c	31.746 \pm 1.376 ^b	9.491 \pm 0.050 ^b	18.572 \pm 0.341 ^b
FOS-EBSG17	23.604 \pm 0.582 ^d	24.775 \pm 2.059 ^a	9.900 \pm 0.657 ^b	15.238 \pm 0.833 ^a

Different letters within the same column show significant differences ($p < 0.05$). TB: Traditional biscuit. FOS-EBSG0: Reduced sugar biscuit with no extruded brewers' spent grain. FOS-EBSG8: Reduced sugar biscuit with 8 % extruded brewers' spent grain. FOS-EBSG17: Reduced sugar biscuit with 17 % extruded brewers' spent grain.

greater addition of EBSG in the biscuits' formulation favored their potential protective activity towards oxidative stress. This might be due to the higher ($p < 0.05$) content of phenolic compounds found in its intestinal digest. In fact, a significant ($p < 0.05$) and negative Pearson correlation ($r = -0.55$) was found between total phenolic content of biscuits (Table 3) intestinal digests and ROS formation in IEC-6. Additionally, Schettino et al (Schettino et al., 2021) found the lowest level of intracellular ROS when Caco-2 cells were incubated in presence of digested pasta made with a 15 % of fermented BSG. However, Merten et al (Merten et al., 2022) found out limited the antioxidant potential of digested bread made with BSG in Caco-2 cell models.

Nevertheless, all the formulated biscuits exhibited certain level of ROS formation inhibition. Therefore, it seemed that other components from the food matrix apart from EBSG favored their antioxidative properties. This may correspond to gluten hydrolysates. It has been previously reported that enzymatic gluten hydrolysates from wheat improved the overall antioxidant capacity in peripheral blood mononuclear cells (Cruz-Chamorro et al., 2020).

3.3.3.2. Anti-inflammatory properties. Results for anti-inflammatory properties of biscuits' intestinal digests are presented in Fig. 4. Only cells treated with biscuits containing 17 % EBSG intestinal digests presented significant ($p < 0.05$) differences in the level of NO formed compared to the control. This effect may be associated to the higher ($p < 0.05$) phenolic content presented in its intestinal digest as a negative Pearson Correlation ($p < 0.001$, $r = -0.75$) was found between the total phenolic content of biscuits digested extracts and the concentration of NO formed. Therefore, biscuits formulated with 17 % EBSG presented enough bioaccessible bioactive compounds concentration to exert an anti-inflammatory effect. This may be important in preventing certain non-communicable diseases, as there is close link between inflammation and many chronic health conditions including diabetes, metabolic syndrome, cardiovascular disease, cancer, inflammatory bowel disease, within others (Zhong & Shi, 2019). Nevertheless, Merten et al (Merten et al., 2022) did not find anti-inflammatory properties for bread made with BSG in their Caco-2 cell model. Authors attributed these results to short time cell exposure to inflammatory stimulation, to the cell line used as Caco-2 cells on their own tend to be less responsive to inflammatory stimuli and to the presence of bile salts or nitrites/ nitrates in digests causing further inflammation.

3.3.3.3. Glucose absorption: Antidiabetic properties. Glucose absorption of biscuits' intestinal digests was evaluated through a cell model employing IEC-6. Results for their areas under the curve (AUC) are shown in Table 7. As expected, due to their lower bioaccessible glucose, EBSG biscuits exhibited lower ($p < 0.05$) glucose absorption than reduced sugar biscuit without EBSG (Table 4). No significant differences were observed between reduced sugar biscuits without addition of EBSG AUC and the control AUC which contained the same amount of free glucose. Similarly, no differences ($p < 0.05$) were found between the glycemic index of biscuits containing 1.6 g of FOS and 1.9 g of spent coffee fibre compared to the control (Campos-Vega, R., Arreguín-Campos, A., Cruz-Medrano & del Castillo, M.D, 2020).

Regarding the glycemic response caused by the ingestion of the analyzed biscuits, by a qualitative approach (Stowell, Bornet, & Jardy-gennetier, 2007), reduced sugar biscuits without EBSG would be categorized as high glycemic; i.e. 45 % of its content corresponds to digestible starch (Table 2). On the other hand, it would be expected that EBSG biscuits presented a lower glycemic response. These trends are in accordance with their glucose absorption AUC. FOS-EBSG8 and FOS-EBSG17's AUC were 12.79 % and 18.61 % lower than FOS-EBSG0's AUC, respectively. Therefore, it would be expected that biscuits supplemented with EBSG induced modest to clinically important benefits on risk factors for certain chronic diseases such as diabetes (Stowell et al., 2007), in comparison to reduced sugar biscuits without EBSG. Nevertheless, to

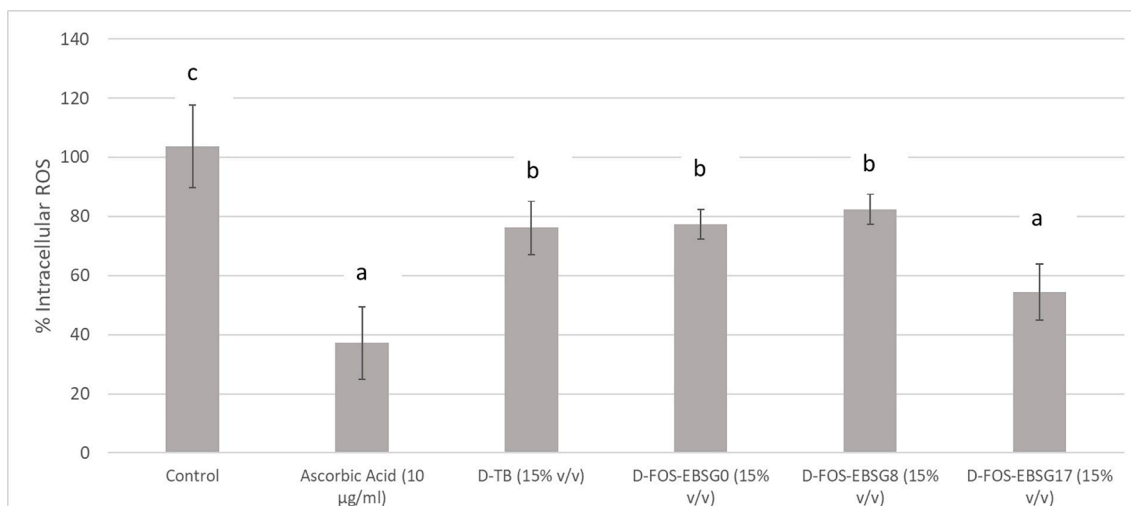


Fig. 3. Intracellular reactive oxygen species (ROS) formation under physiological conditions in IEC-6. Different letters show significant differences ($p < 0.05$). D-TB: Tradition biscuit intestinal digest. D-FOS-EBSG0: Reduced sugar biscuit without extruded brewers' spent grain intestinal digest. D-FOS-EBSG8: Reduced sugar biscuit with 8 % extruded brewers' spent grain intestinal digest. D-FOS-EBSG17: Reduced sugar biscuit with 17 % extruded brewers' spent grain intestinal digest.

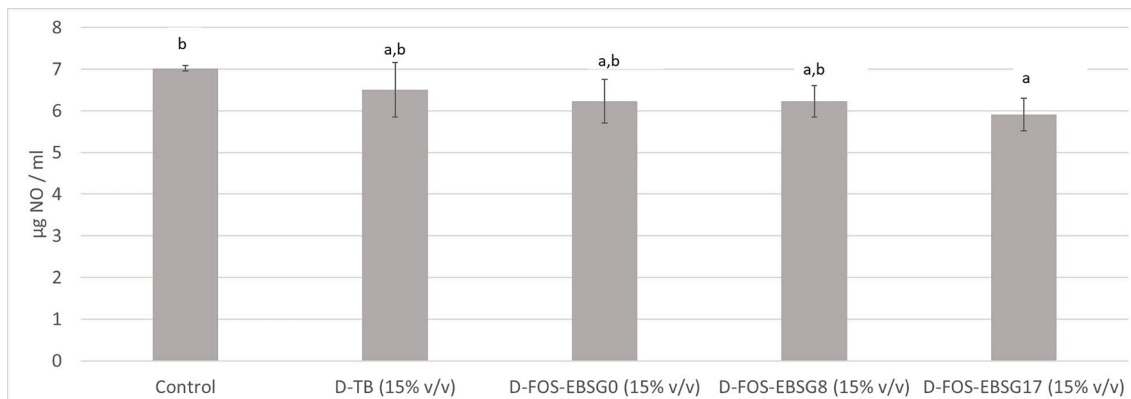


Fig. 4. NO (nitric oxide) formation ($\mu\text{g}/\text{mL}$) in RAW 264.7 induced by LPS ($1 \mu\text{g}/\text{mL}$). Different letters show significant differences ($p < 0.05$). D-TB: Tradition biscuit intestinal digest. D-FOS-EBSG0: Reduced sugar biscuit without extruded brewers' spent grain intestinal digest. D-FOS-EBSG8: Reduced sugar biscuit with 8 % extruded brewers' spent grain intestinal digest. D-FOS-EBSG17: Reduced sugar biscuit with 17 % extruded brewers' spent grain intestinal digest.

Table 7

Area under the curve (AUC) of glucose intestinal absorption.

	AUC Glucose absorption ($\text{mM} \times \text{min}$)
Glucose (2 mM)	38.168 ± 1.729^b
D-FOS-EBSG0 (15 % v/v)	36.375 ± 0.587^b
D-FOS-EBSG8 (15 % v/v)	31.722 ± 1.196^a
D-FOS-EBSG17 (15 % v/v)	29.604 ± 0.407^a

Data expressed as mean \pm standard deviation. Different letters within the same column show significant differences ($p < 0.05$). D-FOS-EBSG0: Reduced sugar biscuit without extruded brewers' spent grain intestinal digest. D-FOS-EBSG8: Reduced sugar biscuit with 8 % extruded brewers' spent grain intestinal digest. D-FOS-EBSG17: Reduced sugar biscuit with 17 % extruded brewers' spent grain intestinal digest.

establish whether there are significant differences in the glycemic index of the tested biscuits, *in vivo* studies should be carried out.

Additionally, a steadier glucose absorption was observed for FOS-EBSG17 than for FOS-EBSG0 (Fig. 5). No acute glucose peak was observed for FOS-EBSG17 glucose absorption pattern over time, while FOS-EBSG0 exhibited a glucose absorption peak in 30 min and 60 min, which was maintained over time ($p > 0.05$). Moreover, FOS-EBSG17 presented a lower glucose absorption at 60 min ($p < 0.05$) than FOS-

EBSG0. FOS-EBSG17 maximum glucose absorption level was achieved at 45 min and there were no significant differences with the glucose levels registered at the following times ($p > 0.05$). This pattern corresponds to low glycemic foods, which are classified as being digested and absorbed slowly (Niwano et al., 2009). Moreover, this behavior might be translated into an increased short term satiety (Niwano et al., 2009; Stowell et al., 2007), compared to FOS-EBSG0 ingestion.

3.4. Colonic fermented digests

3.4.1. Phenolic compounds characterization

No correlation was observed between the addition of EBSG and the phenolic compounds released during colonic fermentation when measured by Folin-Ciocalteu (Table 3). However, results shown in Supplementary material (Fig. S1c, Table S3) suggested that biscuits without EBSG had a higher content of the identified phenolic compounds compared to EBSG biscuits in their colonic fermented digests.

3.4.2. *In vitro* health promoting properties of biscuits' colonic fermented digests

3.4.2.1. Antioxidant properties. Colonic fermented digest of the biscuit containing the highest amount of EBSG presented the lowest ($p < 0.05$)

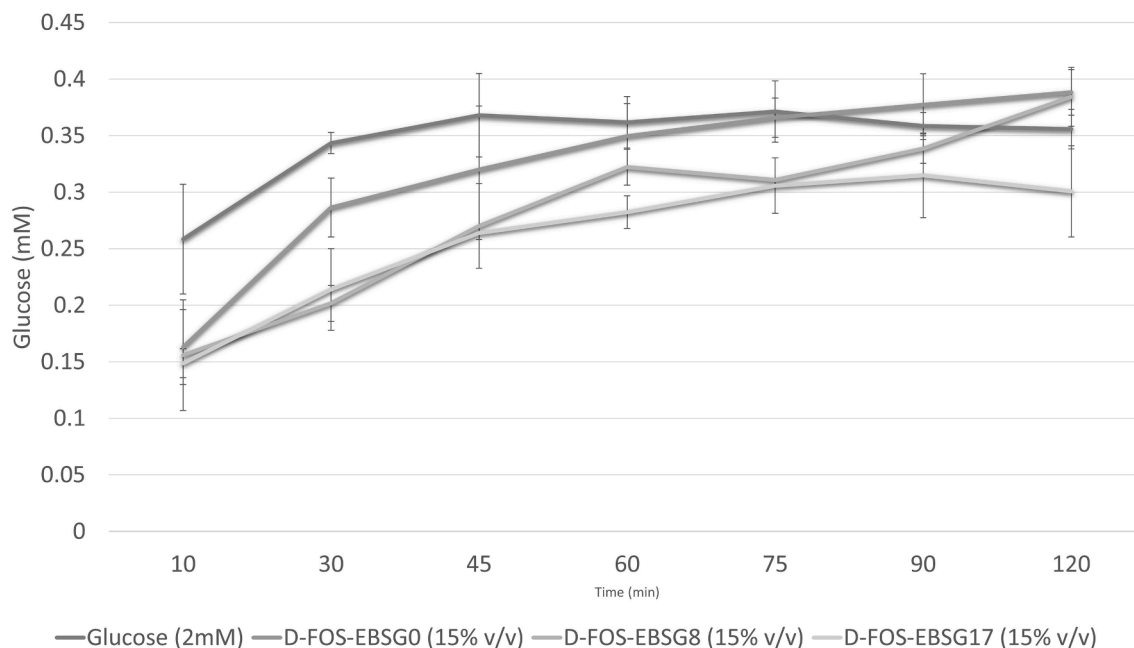


Fig. 5. Time course change in glucose absorption in rat intestinal epithelial cells (IEC-6). Data was obtained in duplicate and results are expressed as mean \pm standard deviation. D-TB: Traditional biscuit intestinal digest. D-FOS-EBSG0: Reduced sugar biscuit without extruded brewers' spent grain intestinal digest. D-FOS-EBSG8: Reduced sugar biscuit with 8 % extruded brewers' spent grain intestinal digest. D-FOS-EBSG17: Reduced sugar biscuit with 17 % extruded brewers' spent grain intestinal digest.

antioxidant capacity when measured by ABTS and ORAC (Table 6). This may correspond to results presented in Fig. S1c: less amount of identified phenolic compounds were registered in colonic fermented digests of biscuits with 17 % EBSG. In fact, a significant positive Pearson Correlation was found between the biscuits colonic fermented digests total phenolic content measured by Folin-Ciocalteu and their antioxidant capacity measured by ABTS ($p < 0.01$, $r = 0.50$) and ORAC ($p < 0.05$, $r = 0.66$).

Furthermore, a higher ($p < 0.05$) antioxidant capacity was obtained during colonic fermentation compared to oral-gastrointestinal digestion, except for FOS-EBSG17 whose intestinal and colonic fermented digest antioxidant capacity did not differ significantly ($p > 0.05$) when measured by ABTS (Table 6). This suggests a higher content of phenolic compounds with HAT mechanism of action may have been released during colonic fermentation of biscuits containing 17 % EBSG, compared to its intestinal digests. In the same line, no significant differences ($p > 0.05$) were found in the antioxidant capacity measured by ABTS of colonic and intestinal digests obtained from biscuits with 10 % defatted sesame flour (Lucini Mas et al., 2022).

3.4.2.2. Fermentability of dietary fiber: Promoting intestinal health. Short-chain fatty acids (SCFAs) formed as end products of fecal microbial metabolism of indigestible carbohydrates (FOS and dietary fiber from EBSG) composing the biscuits are presented in Table 8. Acetic:propionic:butyric ratios were 41:32:27, 40:32:28, 40:30:30, 40:29:30 for TB, FOS-EBSG0, FOS-EBSG8 and FOS-EBSG17, respectively. Significantly higher ($p < 0.05$) concentrations of butyric acid were found in biscuits with FOS compared to TB. It has previously been reported that FOS promotes butyrate production (Ashaolu, 2020; Roupar et al., 2022). Butyric acid is a major oxidative fuel for colonocytes and it has been thought to have important properties in the prevention of colonic cancer (Williams, Grant, Gidley, & Mikkelsen, 2017).

Biscuits with 17 % EBSG had lower ($p < 0.05$) content of propionic acid and higher ($p < 0.05$) contents of valeric and caproic acid than reduced sugar biscuit without EBSG. These metabolites production was previously found after EBSG colonic fermentation (Gutierrez-Barrutia et al., 2022). The formation of valeric acid may be associated to the

Table 8

Short-chain fatty acids, organic acids and sugars present in biscuits' colonic fermented digests.

	Short-chain Fatty Acids (SCFAs) (mM)			
	F-TB	F-FOS-EBSG0	F-FOS-EBSG8	F-FOS-EBSG17
Acetic acid	13.904 \pm 0.722 ^{a,D}	14.762 \pm 0.906 ^{a,D}	13.735 \pm 0.371 ^{a,D}	13.116 \pm 0.675 ^{a,C}
Propionic acid	10.725 \pm 0.802 ^{a,b,C}	11.723 \pm 0.074 ^{b,C}	10.167 \pm 0.043 ^{a,b,C}	9.525 \pm 0.060 ^{a,B}
Butyric acid	8.996 \pm 0.224 ^{a,B}	10.067 \pm 0.411 ^{b,C}	10.059 \pm 0.241 ^{b,B}	9.921 \pm 0.414 ^{b,B}
Isobutyric acid	0.144 \pm 0.007 ^{a,b,A}	0.125 \pm 0.014 ^{a,A}	0.175 \pm 0.005 ^{b,A}	0.261 \pm 0.004 ^{c,A}
Isovaleric acid	0.399 \pm 0.090 ^{a,b,A}	0.364 \pm 0.077 ^{a,A,B}	0.577 \pm 0.041 ^{b,c,A}	0.752 \pm 0.084 ^{c,A}
Valeric acid	0.258 \pm 0.178 ^{a,A}	0.309 \pm 0.207 ^{a,B}	0.804 \pm 0.147 ^{a,A}	1.511 \pm 0.294 ^{b,A}
Caproic acid	0.247 \pm 0.013 ^{a,A}	0.277 \pm 0.080 ^{a,A,B}	0.404 \pm 0.003 ^{a,A}	0.699 \pm 0.051 ^{b,A}
Total	34.556 \pm 1.460 ^a	37.164 \pm 1.040 ^a	36.314 \pm 0.836 ^a	36.285 \pm 1.336 ^a
Sugars (μM)	F-TB	F-FOS-EBSG0	F-FOS-EBSG8	F-FOS-EBSG17
Arabinose*	0.705 \pm 0.674 ^{a,A}	0.460 \pm 0.071 ^{a,A}	1.281 \pm 0.320 ^{a,A}	3.535 \pm 0.412 ^{b,A}

Different letters within the same line (lower case) and column (capital letters) show significant differences ($p < 0.05$). F-TB: Tradition biscuit colonic fermented digest. F-FOS-EBSG0: Reduced sugar biscuit without extruded brewers' spent grain colonic fermented digest. F-FOS-EBSG8: Reduced sugar biscuit with 8 % extruded brewers' spent grain colonic fermented digest. F-FOS-EBSG17: Reduced sugar biscuit with 17 % extruded brewers' spent grain colonic fermented digest.

* Arabinose and xylose coelute in the same peak of the chromatogram and were quantified as arabinose.

fermentation of the hemicellulose forming BSG (Lynch et al., 2016). It was found that diets rich in dietary fiber (xylans, cellulose) from cereals and nonanimal proteins, presented a higher content of valeric acid (Filippo, Cavalieri, Di, Ramazzotti, & Baptiste, 2010). Besides, a significant higher ($p < 0.05$) content of xylose-arabinose was detected in the colonic fermented digest of biscuit containing 17 % EBSG compared to the rest of the analyzed samples (Table 8). In addition, valeric acid formation is also associated to the microbial metabolism of proline (Batta et al., 2020): a major nonessential amino acid in EBSG (Gutiérrez-Barrutia et al., 2022). Valeric acid energizes intestinal epithelium growth and is involved in the prevention of cancer and cardiometabolic diseases by inhibiting histone deacetylase (Yuille, Reichardt, Panda, Dunbar, & Mulder, 2018).

Moreover, the presence of EBSG in biscuits formulation contributed to the formation of branched chain fatty acids (BCFAs) as isovaleric and isobutyric acid (Table 8). These metabolites were previously reported after EBSG colonic fermentation (Gutiérrez-Barrutia et al., 2022) are attributed to the microbial fermentation of valine and leucine (Batta et al., 2020). BCFAs help to regulate electrolytes production and absorption, as well as transmit neural signals in the nervous system (Ashaolu, 2020).

4. Conclusions

The present study provided evidence on nutrients and bioactive compounds released during *in vitro* digestion process of reduced sugar biscuits with extruded brewers' spent grain and fructooligosaccharides. Biscuits were subjected to an *in vitro* simulation of humans' oral-gastro-intestinal digestion and colonic fermentation. Formulated biscuits met the standard for "reduced sugar", "high in dietary fiber" nutrition claims, while biscuits containing 17 % of extruded brewers' spent grain also complied with the nutrition claim "source of protein", following the European legislation (CE N° 1924/2006). After oral-gastrointestinal digestion biscuits containing extruded brewers' spent grain presented lower ($p < 0.05$) bioaccessible glucose levels compared to biscuits without extruded brewers' spent grain. Thus, improved antidiabetic properties were found for biscuits with extruded brewers' spent grain: lower ($p < 0.05$) levels of glucose absorption were determined. The addition of 17 % of extruded brewers' spent grain significantly ($p < 0.05$) improved biscuits *in vitro* antioxidant and anti-inflammatory properties. Regarding colonic fermentability of biscuits, fructooligosaccharides addition caused higher contents of butyric acid, while extruded brewers' spent grain contributed to a higher content of valeric acid formed as metabolite. The combined addition of 17 % extruded brewers' spent grain and 15.2 % fructooligosaccharides outcome the best potential nutritional value and potential health promoting properties among biscuits studied in the present research. This formulation may contribute to reduce the prevalence of non-communicable chronic diseases and play an important role in the development of nutrition security while consumer latest demands are met.

5. Institutional review Board statement

The study was conducted according to the guidelines of the Declaration of Helsinki. Ethical approval for the involvement of human subjects in this study was granted by the Institutional Review Board (or Ethics Committee) of CSIC (protocol code 174/21, approved 2/2/22).

6. Informed consent statement

Informed consent was obtained from all subjects involved in the study.

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CRediT authorship contribution statement

Maria Belen Gutierrez-Barrutia: Investigation, Formal analysis, Data curation, Methodology, Visualization, Writing - original draft, Writing - review & editing. **Sonia Cozzano:** Writing - review & editing. **Patricia Arcia:** Writing - review & editing. **Maria Dolores del Castillo:** Funding acquisition, Methodology, Supervision, Conceptualization, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data presented in this study are available in the present article and in the supplementary material provided.

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Appendix A. Supplementary materials

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2023.113160>.

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