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## Nutritional profiling, fiber content and *in vitro* bioactivities of wheat-based biscuits formulated with novel ingredients†

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This study evaluated the nutritional profile and fiber content of innovative formulations of wheat-based biscuits enriched with chia seeds, carob flour and coconut sugar. The *in vitro* antioxidant, cytotoxic, anti-inflammatory and antimicrobial activities were also investigated to understand the potential health advantages of the incorporation of these new ingredients. The novel biscuits demonstrated significant improvements in protein and mineral content, with increases of 50% and 100% in chia biscuits, and up to 20% and 40% in carob biscuits, respectively. Fiber also notably increased, particularly in samples containing 10% carob flour, which increased four times as compared to wheat-based samples. The new ingredients exhibited antibacterial and antifungal activity, particularly against *Yersinia enterocolitica* (minimum inhibitory concentration 1.25 mg mL<sup>-1</sup> in coconut sugar) and *Aspergillus fumigatus* (minimum inhibitory concentration/minimum fungicidal concentrations 2.5/5 mg mL<sup>-1</sup> in chia seeds). However, the final biscuits only displayed antifungal properties. Carob flour and chia seeds had a remarkably high capacity to inhibit the formation of TBARS and promoted greater antioxidant activity in biscuit formulations, with EC<sub>50</sub> values decreasing from 23.25 mg mL<sup>-1</sup> (control) to 4.54 mg mL<sup>-1</sup> (15% defatted ground chia seeds) and 1.19 mg mL<sup>-1</sup> (10% carob flour). Only chia seeds exhibited cellular antioxidant, anti-inflammatory and cytotoxic activity, attributes that were lost when seeds were added into the biscuits. These findings highlight the potential health benefits of these ingredients, particularly when incorporated in new wheat-based formulations.

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## 1. Introduction

The use of alternative ingredients in traditional food formulations is a common practice that results in novel and appealing organoleptic experiences while maintaining or improving a healthy and nutritious balanced food. Increased consumer awareness of healthy nutrition imposes the need of a new market of innovative products that provide nutritional benefits and promote good health. In addition, the incorporation of health-promoting components into bakery products with a

focus on circular economy and sustainable food production systems is also being explored.<sup>1</sup> The bakery industry has responded to this demand by developing a wide range of products that incorporate alternative ingredients. These products often include the addition or substitution of wheat, either partially or completely, with seeds (chia, sesame, quinoa), legumes (lentils, chickpeas), vegetables (apple, tomato), spices (cinnamon, cloves) different types of cereals (spelt, rye) and pseudocereals (buckwheat). This has resulted in a variety of baked goods, many of them suitable for consumers with gluten intolerance and celiac disease, while also providing added nutritional benefits.<sup>2,3</sup> The trend of incorporating alternative ingredients in bakery products is particularly noticeable in the biscuit industry. Traditional wheat-flour biscuits made with sugars and fats are being replaced with innovative recipes aimed at providing consumers with low-sugar, gluten-free, high-fiber, and mineral-enriched alternatives, among other health benefits.<sup>4</sup>

Chia seed (*Salvia hispanica* L.) is characterized by its high content of dietary fiber (18–40%) and polyunsaturated fatty acids, especially from the ω-3 group, with α-linolenic acid

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being the most representative (60% of the total fatty acids). Additionally, it is a source of proteins (15–24%), carbohydrates (26–41%), and minerals (4–6%).<sup>5,6</sup> Compared to traditional crops such as wheat, corn, rice, and oats, chia has a higher content of protein, fiber, and lipids. Chia also boasts bioactive compounds with strong antioxidant activity, including gallic, caffeic, chlorogenic, cinnamic, or ferulic acid.<sup>6</sup> It also shows a high water absorption capacity, resulting in a clear gel known as chia mucilage. These properties make chia a functional food with remarkable nutritional characteristics, ideal for use in the food industry as a foam-stabilizing agent, suspending agent, emulsifier, adhesive, or binder with water-holding capacity.<sup>5</sup> As such, incorporating chia into certain food formulations is desirable from both a technological and nutritional perspective.<sup>7–9</sup>

Carob flour is obtained from the carob pod of the leguminous tree *Ceratonia siliqua* L., by dehulling and deseeding the pulp, which is then oven dried and mechanically milled.<sup>10</sup> Despite carob pulp being mainly used for animal feed and the production of sugar syrup,<sup>11</sup> it has recently been recognized as an important source of nutrients and essential elements for human metabolism, healthy growth and disease prevention, such as dietary fiber, polyphenols and minerals, while having low amounts of fat.<sup>12</sup> Moreover, it is gluten-free, which makes carob flour an excellent ingredient for developing new food products with enhanced nutritional value and a suitable alternative for people with celiac disease or gluten intolerance.<sup>13–15</sup> For this reason, carob flour is increasingly being used in bakeries,<sup>12</sup> particularly in Mediterranean countries that are facing issues related to climate change.

Reducing the sugar content is another issue for new food formulations. In 2015, the World Health Organization (WHO) recommended that the consumption of added sugars should be reduced to less than 10% of total caloric intake.<sup>16</sup> Cereals and their by-products are the main sources of added sugar, accounting for 19.1% of the total dietary sugar intake.<sup>17</sup> Therefore, it is crucial to consider alternatives to sucrose, such as carob flour and other sweeteners (*i.e.* coconut sugar), which can be added to cereal-based foods and can provide healthier alternatives to combat obesity and other diet-related health issues.<sup>18,19</sup>

In recent years, several investigations have been focused on cereal-based foods formulated with chia and carob flour and there is no information for coconut sugar. The majority of these studies have assessed the psychochemical attributes and consumer acceptability of the final products, including textural and sensorial analysis.<sup>8,9,14,15,20,21</sup> Nevertheless, the researches of the nutritional changes in novel formulations containing carob, chia and coconut sugar are limited or even nonexistent. In this context, the aim of this study was to evaluate the nutritional profile and the fiber content of new formulations of wheat-based biscuits enriched with novel ingredients such as chia seeds, carob flour, and coconut sugar. The goal was designing a cereal-based product that offers added nutritional and physiological values respect to the traditional wheat-based biscuit. Different biological activities *in vitro*, including anti-

oxidant, cytotoxic and anti-inflammatory were also assessed to determine the health-enhancing effects of the reformulated food. Additionally, the antimicrobial activity of novel ingredients was further investigated to set more insight into their potential use as food preservatives and additives aimed at enhancing the safety and shelf life of biscuits. The findings from this research will provide valuable knowledge regarding the health properties of chia seeds, carob flour and coconut sugar, highlighting their potential applications in the food industry.

## 2. Materials and methods

### 2.1. Standards and reagents

Ampicillin, methicillin and streptomycin were acquired from Fischer Scientific (Janseen Pharmaceutical, Belgium) and ketoconazole was provided by Frilabo (Porto, Portugal). Ellipticine, dexamethasone, quercetin, fatty acid methyl ester (FAME) reference standard mixture 37 (standard 47885-U), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and thiobarbituric acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Porcine (*Sus scrofa*) brains were obtained from animals slaughtered at officially licensed premises. Analytical grade reagents were used for all experiments. Sulfuric acid (98%), n-hexane (95%), petroleum ether, methanol, ethanol and toluene were purchased from Fischer Scientific (Leicestershire, UK). Roswell Park Memorial Institute medium (RPMI 1640), Dulbecco's modified Eagle's medium, fetal bovine serum, Hank's balanced salt solution (HBSS) and trypsin-EDTA (ethylenediaminetetraacetic acid) were provided by Hyclone (Logan, Utah, USA). Malt extract broth and Muller-Hinton broth were from Biolab® (Budapest, Hungary) while blood agar (Sheep blood 7%) was from LiofilChemsrl (Roseto d. Abruzzi (TE), Italy). 2',7'-Dihydrodichlorofluorescein diacetate, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), tris(hydroxymethyl) aminomethane, trichloroacetic acid, dimethyl sulfoxide (DMSO), lipopolysaccharide, and sodium nitrate were also from Sigma-Aldrich (St. Louis, MO, USA). *p*-Iodonitrotetrazolium chloride and sodium sulfate were provided by Panreac Applichem (Barcelona, Spain). Water was treated through a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

### 2.2. Ingredients

Black chia seeds (*Salvia hispanica* L.) originating from Mexico and certified for organic cultivation were purchased from a local market (Madrid, Spain) (Lot C-O-AD18-3-V1, importer EcoAndes Import Export, S.L.). These seeds were subjected to different physical-chemical treatments and the following samples were obtained: (1) ground chia seed (GC): whole seeds were ground using an Ultra-turrax (IKA, T10 basic, Boutersem, Belgique) to obtain a homogeneous mixture and passed through a mesh size sieve of 0.80 mm; (2) defatted ground chia seed (DGC): ground chia seeds were washed twice with hexane (1 : 10, *w/v*) and left to dry for 20 hours at room temp-



erature. Carob flour, coconut sugar, wheat flour and other food-grade ingredients (white sugar, salt and sunflower oil) were purchased from local supermarkets (Madrid, Spain).

### 2.3. Preparation of biscuits

Fourteen biscuits were prepared according to the recipe described in the AACC (American Association of Cereal Chemists) method 10-54<sup>22</sup> (ESI Table 1†). Control biscuit was formulated with wheat flour (130 g), white sugar (35 g), distilled water (30 g), sunflower oil (26 g), sodium bicarbonate (0.8 g), ammonium bicarbonate (0.4 g) and salt (1 g).

- Ground chia biscuits (GC): wheat flour was replaced by ground chia seed, with percentages in the final weight of 5% (GC5), 10% (GC10) and 15% (GC15).

- Defatted ground chia biscuits (DGC): wheat flour was replaced by defatted ground chia seed, with percentages in the final weight of 5% (DGC5), 10% (DGC10) and 15% (DGC15).

- Carob flour biscuits (CF): wheat flour was replaced by carob flour, with percentages in the final weight of 1% (CF1), 5% (CF5) and 10% (CF10).

- Coconut sugar biscuit (CS): 100% of white sugar was replaced by coconut sugar (CS100).

- Carob flour and coconut sugar biscuits: wheat flour was replaced by carob flour, with percentages in the final weight of 1% (CF1), 5% (CF5), and 10% (CF10), and 100% of white sugar was replaced by coconut sugar (CS100).

The total amount of solids in the dough remained the same. Ingredients were thoroughly mixed and the dough was rolled out to disks with the diameter of 6.5 cm and the thickness of 2 mm, and baked at 180 °C for 22 minutes in a convention oven (Memmert UFE 400, Germany).

### 2.4. Proximal nutritional composition

The proximal composition was determined in ingredients and biscuits separately. For this purpose, the official analysis methodologies AOAC (Association of Official Analytical Chemists) were used.<sup>23</sup> Moisture was determined gravimetrically by desiccation in an oven at 105 °C to constant weight, according to AOAC 984.25. The crude protein (N × 6.25) was evaluated using the macro-Kjeldahl method (AOAC 991.02). Fat content was determined by Soxhlet extraction with petroleum ether according to AOAC 989.05. The total dietary fiber (TDF) content was determined by the AOAC 985.29 method, using the Kit TDF100A (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Total mineral content was determined as ash by incineration at 550 °C (AOAC 935.42). Results are expressed as g/100 g of fresh sample. Total carbohydrates (including fiber) were calculated by difference. Energy was estimated using the following conversion factors: 4 kcal g<sup>-1</sup> for proteins and carbohydrates (excluding fiber) and 9 kcal g<sup>-1</sup> for fat and results were expressed in kcal/100 g.<sup>24</sup>

### 2.5. Analysis of soluble sugars

Soluble sugars were analyzed according to Pinela *et al.*<sup>25</sup> Samples were spiked with the internal standard melezitose (25 mg mL<sup>-1</sup>) and extracted for 90 min with ethanol/water

80 : 20 (v/v) at 80 °C. The analysis was performed in a high-performance liquid chromatography (HPLC) system coupled to a refractive index (RI) detection. Chromatographic separation was achieved with a Eurospher 100-5 NH<sub>2</sub> column (4.6 × 250 mm, 5 µm, Knauer). The mobile phase was acetonitrile/water 70 : 30 (v/v). Soluble sugars were identified by comparison with pure standards and quantified based on the internal standard concentration. The results were expressed in g/100 g fresh sample.

### 2.6. Analysis of fatty acids profile

Fatty acid methyl esters (FAMEs) were prepared from oils derived from the determination of fat content in both ingredients and biscuits. The transesterification reaction was carried out by mixing the oils (500 µL) with a catalytic solution of methanol, sulfuric acid, and toluene (2 : 1 : 1, v/v/v) (5 mL). The mixture was incubated overnight at 50 °C and 600 rpm. After that, phase separation was achieved by adding 3 mL of water and 3 mL of diethyl ether to the mixture and vortexing for 30 seconds. The FAMEs were recovered from the upper layer and mixed with sodium sulfate. The samples were then filtered using 0.22 µm nylon filters and diluted 1/10 in diethyl ether and stored at -20 °C until further analysis.

FAMEs analysis was conducted using a gas chromatograph (GC) equipped with a flame ionization detector (FID), a split/splitless injector, and a capillary column (30 m × 0.32 mm ID × 0.25 µm, Macherey-Nagel). The identification of fatty acids was performed by comparing the relative retention times of FAME peaks obtained from the oil extracted from samples with a reference standard FAME mixture. The specific details of the chromatography separation and determinations were previously described by Reis *et al.*<sup>26</sup> The results were expressed in relative percentage (%).

### 2.7. Evaluation of *in vitro* biological activities

For the extract preparation, 1 g of both ingredients and biscuits was used. Firstly, samples were macerated at room temperature with ethanol/water solution (80 : 20, v/v; 30 mL). The hydroethanolic extract was filtered using a filter (Whatman No. 4) and the retentate was extracted again using the same procedure. The ethanolic fraction of the extracts was evaporated under reduced pressure in a rotary evaporator (Büchi R-210, Flawil, Switzerland) and the aqueous fraction was frozen and lyophilized (Freezone 4.5, Labconco, Kansas City, MO, USA). The lyophilized extracts were re-dissolved: (i) in culture medium at 20 mg mL<sup>-1</sup> for the evaluation of antimicrobial activity; (ii) in distilled water at 8 mg mL<sup>-1</sup> for the evaluation of cytotoxic and anti-inflammatory activity and at 2 mg mL<sup>-1</sup> for cellular antioxidant activity evaluation; and (iii) in a hydroethanolic solution (ethanol/water; 80 : 20, v/v) at 200 mg mL<sup>-1</sup> for TBARS inhibition assay. Stock solutions of lyophilized extracts were diluted successively to obtain the appropriate range of working concentrations.

**2.7.1. Antimicrobial activity.** Methods described by Pires *et al.*<sup>27</sup> and Heleno *et al.*<sup>28</sup> were employed for antimicrobial assays. Eight bacterial strains were tested for antibacterial



activity. *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* serotype *Enteritidis* (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610), *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111) and *Staphylococcus aureus* (ATCC 11632). Two fungal strains were tested for antifungal activity: *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404). Commercial strains of these micro-organisms were acquired from Frilabo (Porto, Portugal). Streptomycin and ampicillin were used as a positive control for antibacterial assays, except for *S. aureus*, for which methicillin was employed. Ketoconazole was selected as positive control for the antifungal assays.

Gram-positive bacteria (*B. cereus*, *L. monocytogenes*, *S. aureus*) were incubated in fresh blood agar containing 7% sheep blood, while Gram-negative bacteria (*E. cloacae*, *E. coli*, *P. aeruginosa*, *S. enterica*, *Y. enterocolitica*) were incubated in Muller Hilton agar. Both types of bacteria were incubated at 37 °C for 24 hours to maintain the exponential growth phase. Bacterial suspensions were prepared at a concentration of  $1.5 \times 10^6$  CFU mL<sup>-1</sup>. Malt agar plates were used for the micromyces (fungal strains) and they were incubated at 25 °C for 72 hours. After that, fungal spores were recovered from the agar surface using sterile 0.85% saline solution containing 0.1% Tween 80 (v/v). Fungal spore suspensions were adjusted to a concentration of  $1.0 \times 10^5$  UFC mL<sup>-1</sup>.

Results were presented as minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentrations (MFC) and expressed in mg mL<sup>-1</sup>.

**2.7.2. Antioxidant activity.** For the antioxidant activity evaluation, two different *in vitro* assays were used: the cell-based assays of thiobarbituric acid reactive substances (TBARS) formation inhibition and the cellular antioxidant activity (CAA). The capacity of the extracts to inhibit the formation of TBARS was assessed according to Lockowandt *et al.*<sup>29</sup> using porcine brain tissue as oxidizable biological substrates. Trolox was used as a positive control. The results were expressed as EC<sub>50</sub> values (mg mL<sup>-1</sup>), which translate the extract concentration responsible for 50% of antioxidant capacity.

For the cellular antioxidant activity assessment, the method described by de la Fuente *et al.*<sup>30</sup> was followed. Extracts at various concentrations (ranging from 500 to 2000 µg mL<sup>-1</sup>) were prepared in H<sub>2</sub>O. The extracts were incubated with a murine macrophage cell line (RAW 264.7); obtained from the European Collection of Authenticated Cell Cultures (ECACC) at 37 °C for 1 hour. After incubation, the medium was discarded, and the cells were washed twice with 100 µL of HBSS. Then, 100 µL of AAPH (600 µM) was added to the cells. The reaction was carried out using a plate reader Bioteck FLX800 (Bio-Tek Instruments, Inc., Winooski, VT, USA) with fluorescence filters set at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Fluorescence values were recorded every 5 minutes over a period of 1 hour, and the differences in the areas under the curve (AUC) were calculated. Quercetin standard

was used as a positive control. The CAA values were determined using the equation CAA unit =  $100 - (\int \text{AUCs} / \int \text{AUCc}) \times 100$ , where  $\int \text{AUCs}$  represents the integrated area under the sample fluorescence *versus* time curve, and  $\int \text{AUCc}$  represents the integrated area from the control curve. The results were expressed as a percentage of inhibition of the oxidation reaction.

**2.7.3. Anti-inflammatory activity.** Both ingredient and biscuit extracts at concentrations of 6.25–400 µg mL<sup>-1</sup> (in water) were tested for the determination of anti-inflammatory activity. Briefly, the stimulation of murine macrophages RAW 264.7 cell line was measured by means of nitric oxide (NO) production by using the Griess Reagent System kit (Promega, WI, USA), as previously described by Souilem *et al.*<sup>31</sup> Dexamethasone was used as positive control and the results were expressed as IC<sub>50</sub> values, in µg mL<sup>-1</sup>.

**2.7.4. Cytotoxic activity.** Both ingredient and biscuit extracts at concentrations of 6.25–400 µg mL<sup>-1</sup> (in water) were used for the determination of cytotoxic activity against different human tumor cell lines: gastric adenocarcinoma, AGS; colon adenocarcinoma, CaCo-2; breast adenocarcinoma, MCF-7; and non-small cell lung cancer, NCI-H460. AGS and Caco-2 cell lines were obtained from ECACC, whereas the MCF-7 and NCI-H460 cell lines were purchased from the Leibniz-Institute DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Ellipticine was selected as positive control, and the results were expressed as GI<sub>50</sub> values, in µg mL<sup>-1</sup>. Cells were treated according to the protocol described by Mandim *et al.*<sup>32</sup> The sulforhodamine B (SRB) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA) described by de la Fuente *et al.*<sup>30</sup> was applied.

## 2.8. Statistical analysis

All determinations were carried out in triplicate, and results were expressed as the mean  $\pm$  standard deviation (SD). Statistical analysis was performed by using Statgraphics Centurion XV (Herndon, VA, USA). Firstly, to meet the requirements for the performance of analysis of variance (ANOVA) the normality of residuals and homoscedasticity were assessed by conducting the Sapiro-Wilk's and Levene's tests, respectively. Then, one-way ANOVA was carried out to evaluate statistical differences between treatments, followed by Tukey's HSD *post hoc* test. Statistical differences with respect to positive controls were evaluated by student's *t*-test. Differences were considered to be significant at  $p < 0.05$ .

## 3. Results and discussion

### 3.1. Proximal composition of ingredients and biscuit formulations

The proximal composition of the ingredients is presented in Table 1, including (1) wheat flour and white sugar as control (traditional ingredients), (2) chia seeds and carob flour as wheat flour partial replacers and (3) coconut sugar as white sugar replacer. In comparison to wheat flour, ground chia



**Table 1** Proximate composition and energy value of novel ingredients (chia seeds, carob flour and coconut sugar) as compared with traditional ones (wheat flour and white sugar)

	Control Wheat flour	Wheat flour replacers			Control White sugar	Sugar replacer Coconut sugar
		Ground chia seed	Defatted ground chia seed	Carob flour		
Proximate composition (g/100 g fresh sample)						
Protein	9.47 ± 0.31c	21.69 ± 0.25e	18.06 ± 0.14d	5.28 ± 0.07b	nd	0.92 ± 0.44a
Fat	1.16 ± 0.01b	32.03 ± 0.42d	8.21 ± 0.06c	0.42 ± 0.01ab	nd	0.20 ± 0.06a
Total saturated fat	0.30 ± 0.01b	8.36 ± 0.08d	2.49 ± 0.06c	0.13 ± 0.01a	nd	0.11 ± 0.01a
C6:0 (%)	nd	1.10 ± 0.01b	0.40 ± 0.01a	nd	nd	nd
C8:0 (%)	nd	0.50 ± 0.20a	0.70 ± 0.10a	nd	nd	nd
C14:0 (%)	0.20 ± 0.10a	nd	nd	0.40 ± 0.01b	nd	nd
C16:0 (%)	23.10 ± 0.50a	17.60 ± 2.40a	21.30 ± 1.20a	25.30 ± 3.80a	nd	46.70 ± 0.01b
C18:0 (%)	1.60 ± 0.01a	6.90 ± 0.40bc	8.01 ± 0.40c	5.40 ± 0.80b	nd	7.80 ± 0.10c
Total unsaturated fat	0.83 ± 0.03c	22.93 ± 1.15e	5.39 ± 0.07d	0.26 ± 0.02b	nd	0.09 ± 0.01a
C18:1n9 (%)	16.01 ± 0.80b	11.70 ± 0.30a	13.30 ± 0.60a	42.80 ± 0.40d	nd	34.01 ± 0.40c
C18:2n6 (%)	52.30 ± 0.10c	18.60 ± 0.30b	18.20 ± 0.50b	16.20 ± 4.30ab	nd	8.90 ± 0.60a
C18:3n3 (%)	3.40 ± 0.10a	41.30 ± 3.80a	34.20 ± 2.40b	2.70 ± 1.30a	nd	nd
Total carbohydrates	79.41 ± 0.38c	36.19 ± 0.04a	61.33 ± 0.25b	87.70 ± 0.03d	99.97 ± 0.00f	94.12 ± 0.36e
Fiber	3.41 ± 0.42b	25.60 ± 0.31c	31.23 ± 0.42d	35.80 ± 0.51e	nd	1.30 ± 0.06a
Soluble sugars	0.50 ± 0.06a	0.93 ± 0.04a	0.79 ± 0.06a	30.86 ± 0.26b	104.41 ± 0.69d	95.46 ± 1.48c
Fructose	nd	0.14 ± 0.02a	0.11 ± 0.03a	9.04 ± 0.13c	nd	2.42 ± 0.06b
Glucose	nd	nd	nd	2.80 ± 0.05b	nd	4.12 ± 0.14c
Sucrose	0.50 ± 0.06a	0.79 ± 0.01a	0.68 ± 0.03a	19.02 ± 0.09b	104.41 ± 0.69d	88.92 ± 1.29c
Moisture	9.44 ± 0.00f	5.54 ± 0.00d	7.31 ± 0.00e	4.41 ± 0.00c	0.03 ± 0.00a	3.62 ± 0.00b
Total mineral content	0.52 ± 0.06a	4.54 ± 0.12d	4.99 ± 0.05e	2.19 ± 0.03c	nd	1.14 ± 0.05b
Energy value (kcal/100 g)	352.32 ± 0.16b	417.44 ± 2.58c	266.53 ± 0.06a	232.48 ± 1.61a	399.89 ± 0.01b	376.76 ± 0.06b

Results are mean ± standard deviation ( $n = 3$ ). nd: not detected. Different letters in the same line indicate significant differences between samples ( $p < 0.05$ ).

seeds presented significantly higher levels of proteins, fats, dietary fiber, minerals and soluble sugars, while exhibiting lower total carbohydrate content. The primary fatty acid detected in wheat flour was linoleic acid (C18:2n6), accounting for 52.3% of the lipid profile. In contrast, ground chia seeds were mainly composed of linolenic acid (C18:3n3), with a significantly lower contribution from linoleic acid (C18:2n6). These values align with findings from previous studies.<sup>20</sup> Upon the defatting process, the fat content notably decreased from 32.03% in ground seeds to 8.21% in the defatted samples, which resulted in an increase in the levels of most other components. In agreement with Nassef *et al.*,<sup>20</sup> significant differences ( $p < 0.05$ ) were observed in the content of all chemical compositions among ground chia seeds, defatted ground chia seeds, and wheat flour. In the case of carob flour, the most prominent feature was its high fiber and soluble sugar content. Additionally, it showed higher levels of minerals but lower fat content compared to wheat flour, as previously reported by other authors.<sup>13</sup> Carob flour also had a lower protein content compared to wheat; however, it has been described that it contains higher levels of certain essential amino acids such as lysine, histidine, valine and threonine than wheat.<sup>13</sup> This characteristic makes carob a high-quality protein ingredient with important nutritional value. The predominant fatty acid in carob was oleic acid (C18:1n9), again with a significantly lower contribution of the linoleic acid as compared with wheat flour, in accordance with observations from Youssef *et al.*<sup>33</sup> Regarding sweeteners, unlike white sugar, which primarily consists of soluble sugars, coconut sugar pre-

sented protein, fiber and minerals. It had a minimal fat content, with the predominant fatty acid being palmitic acid (C16:0).

Novel formulations of biscuits were prepared with partial or total replacement of traditional ingredients (wheat flour and white sugar) by innovative ingredients (chia seeds, carob flour and coconut sugar) (ESI Table 1†). Chia biscuits were formulated with both defatted and non-defatted ground chia seeds in a range between 5 and 15% of the final weight. Despite chia seeds are allowed as an ingredient in baked goods at concentrations below 10%,<sup>34</sup> experiments were conducted with percentages as high as 15% to gain a deeper understanding of the nutritional composition and *in vitro* bioactivities of the biscuits formulated with these seeds. Similarly, carob flour was added to the biscuits in a range between 1 and 10% of the final weight. As expected, the addition of chia led to an increase in protein, fat, fiber, and mineral content, while concurrently reducing total carbohydrate content without modifying the energy value of the final product (Table 2). This change resulted in an improved nutritional profile when compared to the control sample. The rise in fiber and mineral content was particularly pronounced when defatted seeds were incorporated, resulting in a slight uptick in fat content when they were added at percentages of 10 and 15%. In the case of chia biscuits, the increase in fat content resulted in a positive outcome since chia is abundant in polyunsaturated fatty acids, primarily omega-3 fatty acids (C18:3n3, linolenic acid). These fatty acids are considered essential because the human body cannot synthesize them and they must be obtained through



**Table 2** Proximate composition and energy value of novel biscuits formulated with ground chia seeds (GC), defatted ground chia seeds (DGC), carob flour (CF) and coconut sugar (CS) as compared with traditional biscuits formulated with wheat flour (control)

	Control	GC5	GC10	GC15	DGC5	DGC10	DGC15
Proximate composition (g/100 g fresh sample)							
Protein	5.98 ± 0.32a	7.50 ± 0.10cde	8.30 ± 0.04ef	9.16 ± 0.03f	7.32 ± 0.08bcd	7.98 ± 0.20de	7.94 ± 0.24de
Fat	14.55 ± 0.02ab	16.51 ± 0.08de	18.15 ± 0.23f	20.51 ± 0.39g	14.75 ± 0.24ab	15.67 ± 0.05cd	16.61 ± 0.01e
Total saturated fat	1.82 ± 0.07a	2.15 ± 0.06b	3.86 ± 0.18c	5.84 ± 0.05e	1.93 ± 0.03a	2.04 ± 0.04ab	4.72 ± 0.05d
C6:0 (%)	nd	nd	0.50 ± 0.10a	1.90 ± 0.80b	nd	nd	4.10 ± 1.70c
C8:0 (%)	nd	nd	0.20 ± 0.10a	0.70 ± 0.20b	nd	nd	0.80 ± 0.10b
C14:0 (%)	0.10 ± 0.01a	0.10 ± 0.01a	0.10 ± 0.01	0.20 ± 0.01b	0.10 ± 0.01a	0.10 ± 0.01a	0.20 ± 0.01b
C16:0 (%)	8.50 ± 0.20a	8.70 ± 0.20a	14.01 ± 0.30b	17.60 ± 1.30c	8.80 ± 0.40a	8.60 ± 0.01a	14.30 ± 0.80c
C18:0 (%)	4.10 ± 0.10a	4.20 ± 0.01a	6.50 ± 0.10a	8.10 ± 0.40d	4.20 ± 0.40a	4.20 ± 0.20a	9.01 ± 0.40d
Total unsaturated fat	12.41 ± 0.21bc	14.03 ± 0.79d	13.57 ± 0.21c	11.83 ± 0.20d	12.51 ± 0.23bc	13.26 ± 0.68c	10.45 ± 0.23a
C18:1n9 (%)	29.80 ± 0.10bc	27.30 ± 0.01a	37.01 ± 0.40d	37.10 ± 0.50d	28.60 ± 0.30abc	28.60 ± 0.31abc	24.40 ± 0.90e
C18:2n6 (%)	55.30 ± 0.20d	52.90 ± 0.01d	33.20 ± 1.20c	24.10 ± 3.30b	53.10 ± 0.01d	51.01 ± 1.01d	34.10 ± 2.20a
C18:3n3 (%)	0.20 ± 0.01a	4.80 ± 0.10c	4.60 ± 0.20c	4.00 ± 0.70bc	3.10 ± 0.01b	5.01 ± 0.31c	4.40 ± 0.10a
Total carbohydrates	76.25 ± 0.39g	73.00 ± 0.37cde	70.44 ± 0.39b	67.26 ± 0.47a	74.18 ± 0.02def	72.94 ± 0.12cd	71.99 ± 0.17bc
Fiber	2.63 ± 0.06a	5.80 ± 0.31c	8.13 ± 0.53f	10.83 ± 0.66h	7.47 ± 0.64e	9.57 ± 0.95gh	13.73 ± 1.35j
Soluble sugars	nd	18.90 ± 0.10abcd	19.45 ± 0.15bcd	18.61 ± 0.02abcd	nd	18.69 ± 0.89abcd	19.03 ± 0.17abcd
Fructose	nd	nd	nd	nd	nd	nd	0.14 ± 0.01a
Glucose	nd	nd	nd	nd	nd	nd	0.20 ± 0.01a
Sucrose	19.21 ± 0.01c	18.90 ± 0.10abc	19.45 ± 0.15c	18.61 ± 0.02abc	18.15 ± 0.78abc	18.69 ± 0.89abc	18.69 ± 0.80abc
Moisture	2.10 ± 0.00b	1.29 ± 0.01a	1.36 ± 0.00a	1.05 ± 0.01a	2.34 ± 0.01b	1.68 ± 0.01ab	1.24 ± 0.00a
Total mineral content	1.11 ± 0.05ab	1.69 ± 0.19bcd	1.75 ± 0.21bcd	2.01 ± 0.05cd	1.40 ± 0.14abc	1.73 ± 0.27bcd	2.22 ± 0.06d
Energy value (kcal/100 g)	449.40 ± 0.14c	447.39 ± 0.37c	445.78 ± 0.34c	447.00 ± 1.71c	428.92 ± 1.73b	426.47 ± 0.84b	414.33 ± 0.17a
	Control	CF1	CF5	CF10	CS100	CF1-CS100	CF5-CS100
Proximate composition (g/100 g fresh sample)							
Protein	5.98 ± 0.32a	6.53 ± 0.21abc	6.65 ± 0.09abc	6.26 ± 0.34ab	7.10 ± 0.04abcd	6.92 ± 0.07abcd	6.58 ± 0.00abc
Fat	14.55 ± 0.02ab	14.64 ± 0.08ab	14.80 ± 0.12ab	14.26 ± 0.01a	14.20 ± 0.08a	14.56 ± 0.14ab	15.09 ± 0.12bc
Total saturated fat	1.82 ± 0.07a	1.93 ± 0.08a	1.95 ± 0.05a	1.89 ± 0.01a	1.92 ± 0.04a	2.02 ± 0.03ab	2.28 ± 0.05b
C6:0 (%)	nd	nd	nd	nd	nd	nd	nd
C8:0 (%)	nd	nd	nd	nd	nd	nd	nd
C14:0 (%)	0.10 ± 0.01a	0.1 ± 0.0a	0.1 ± 0.0a	0.1 ± 0.0a	0.1 ± 0.0a	0.1 ± 0.0a	0.1 ± 0.0a
C16:0 (%)	8.50 ± 0.20a	8.6 ± 0.3a	8.4 ± 0.1a	8.4 ± 0.2a	8.8 ± 0.5a	8.9 ± 0.2a	9.2 ± 0.1a
C18:0 (%)	4.10 ± 0.10a	4.5 ± 0.0a	4.7 ± 0.1ab	4.8 ± 0.1ab	4.6 ± 0.2ab	4.9 ± 0.3ab	5.8 ± 0.3bc
Total unsaturated fat	12.41 ± 0.21bc	12.32 ± 0.31bc	12.47 ± 0.27bc	12.02 ± 0.15b	11.94 ± 0.07b	12.14 ± 1.01b	12.42 ± 0.21bc
C18:1n9 (%)	nd	nd	nd	nd	nd	nd	nd
C18:2n6 (%)	30.6 ± 0.2c	30.5 ± 0.0c	30.5 ± 0.4c	30.5 ± 0.3c	30.5 ± 0.3c	28.3 ± 0.1ab	27.7 ± 0.2a
C18:3n3 (%)	53.30 ± 0.20d	53.4 ± 0.2d	53.6 ± 0.4d	53.3 ± 0.6d	52.6 ± 0.1d	53.3 ± 0.5d	56.0 ± 0.2d
Total carbohydrates	0.20 ± 0.01a	0.2 ± 0.0a	0.2 ± 0.0a	0.2 ± 0.0a	0.3 ± 0.1a	0.3 ± 0.1a	0.2 ± 0.0a
Fiber	76.25 ± 0.39g	76.19 ± 0.36g	75.98 ± 0.31fg	75.02 ± 0.49fg	74.98 ± 0.16fg	75.09 ± 0.13fg	74.79 ± 0.12efg
Soluble sugars	nd	nd	nd	nd	nd	nd	nd
Fructose	nd	nd	nd	nd	nd	nd	nd
Glucose	nd	nd	nd	nd	nd	nd	nd
Sucrose	nd	nd	nd	nd	nd	nd	nd
Moisture	19.21 ± 0.01c	2.63 ± 0.06a	2.70 ± 0.06a	5.09 ± 0.02b	9.11 ± 0.14g	2.86 ± 0.10a	6.12 ± 0.01d
Total mineral content	1.11 ± 0.05ab	0.84 ± 0.05a	1.43 ± 0.00ab	3.24 ± 0.02c	15.79 ± 0.00a	16.26 ± 0.24ab	15.91 ± 0.06a
Energy value (kcal/100 g)	449.40 ± 0.14c	449.50 ± 0.42c	441.87 ± 0.70c	416.06 ± 0.09a	1.24 ± 0.04bc	1.27 ± 0.00ab	1.58 ± 0.03bcd
					442.42 ± 1.31c	441.45 ± 0.07c	419.49 ± 0.01ab

Results are mean ± standard deviation ( $n = 3$ ). nd: not detected. Different letters in the same line indicate significant differences between samples ( $p < 0.05$ ).

dietary sources.<sup>7</sup> At this point, it is important to mention that an increase in unsaturated fatty acids could promote lipid oxidation during storage. Therefore, although the chemical modifications of biscuit during storage are not within the scope of the present study, it is relevant to carefully assess the potential impact of lipid oxidations throughout the shelf life in a risk/benefit context. Previous studies conducted by our research group reported an acceleration of lipid oxidation in chia-enriched biscuits, particularly due to the increase in polymerization compounds, which suggests a decreased shelf life of the product by promoting rapid rancidity.<sup>35</sup> Consistent with the results of this study, several researchers have also documented the increase of proteins, fiber, and fats when chia is included in formulations of bakery products.<sup>8,9</sup>

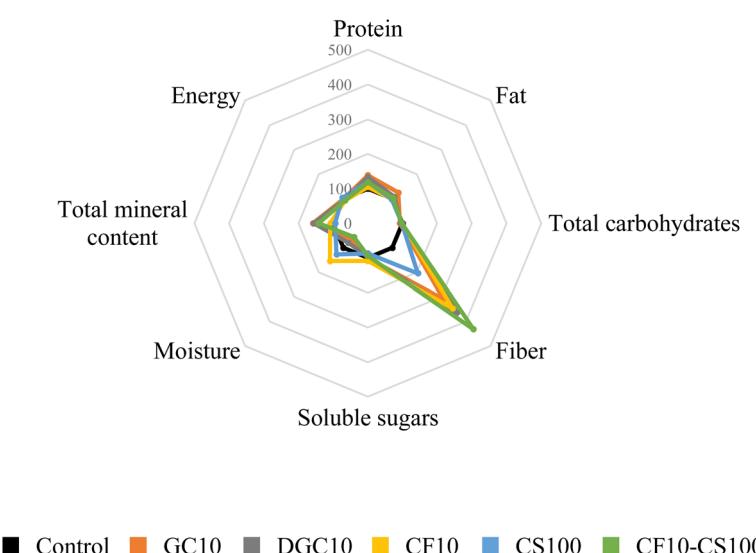
The addition of carob flour fundamentally resulted in a significant increment of the fiber content in biscuits, particularly at substitutions level of 5% and 10% (Table 2). Babiker *et al.*<sup>14</sup> described that a complete replacement of wheat flour with carob flour in cookies led to a significant rise in protein content (from 8.94% to 13.49%) and a decrease in fat content (from 14.20% to 12.30%). However, a 50% substitution with carob flour only exerted residual changes in the composition. In the present study, the protein content reached 9.24%, and the fat content was 14.00%, which closely resembled the levels in the control sample. Despite the proximate composition of the biscuits with carob was very similar to the control biscuit, the notable increase in fiber, even with just 1% incorporation of carob, suggests an improvement for health in this new formulation. This is particularly significant given the well-documented health benefits associated with increased fiber intake. Research, such as the recent review by Ioniță-Mindrigan *et al.*<sup>36</sup> has linked higher fiber consumption to various positive health outcomes, including reduced risk of diabetes, obesity, constipation, as well as a lowered risk of coronary

heart diseases and stroke. The nutritional composition of the biscuits made with carob flour and coconut sugar was remarkably similar to those made with common sugar. However, there was a slight increase in protein, mineral and fiber content in the former, suggesting a mild additional improvement in the nutritional profile compared to the control sample. Given the low-fat content of the flours used, there was not a significant change in the lipid profile that warrants further comment (Table 2).

Fig. 1 depicts the proximal composition and fiber content of both the control biscuit and the new formulations containing chia, carob, and coconut sugar. The formulations with 10% chia and carob were specifically chosen for comparison under similar conditions, adhering to the maximum allowable concentration of chia in baked products.<sup>34</sup> As mentioned before, the novel biscuits exhibited notable improvements in protein and mineral content (up to 50%), and substantially there was a rise in fiber when compared to traditional wheat-based biscuits, particularly in samples containing carob flour (up to four times). These findings underscore the potential health advantages associated with the incorporation of these new ingredients into the reformulated biscuits.

### 3.2. Bioactivities of ingredients and biscuit formulations

**3.2.1. Antimicrobial activity.** Tables 3 and 4 display the results obtained for the antimicrobial activity of the extracts derived from ingredients and biscuit formulations. Results of antibacterial activity are expressed as MIC and MBC, while those of antifungal activity are expressed as MIC and MFC. Regarding ingredients (Table 3), wheat exhibited the lowest antibacterial effect, only inhibiting the growth of *E. Cloacae* and *P. aeruginosa* at concentrations of 20 mg mL<sup>-1</sup>. Chia seeds, carob flour, white sugar and coconut sugar presented antibacterial activity against almost all bacteria tested, with



**Fig. 1** Proximal composition and fiber content of the control biscuit and the new formulations containing ground chia seeds (GC), defatted ground chia seeds (DGC) and carob flour (CF) at 10% and coconut sugar (CS) at 100%.



**Table 3** Antibacterial (MIC/MBC, mg mL<sup>-1</sup>) and antifungal activity (MIC/MFC, mg mL<sup>-1</sup>) of extracts obtained from novel ingredients (chia seeds, carob flour and coconut sugar) as compared with traditional ones (wheat flour and white sugar)

	Wheat	GC	DGC	CF	WS	CS	Positive controls			
Antibacterial activity										
Gram-negative bacteria										
<i>E. cloacae</i>	20/20	20/20	20/20	20/20	>20/20	>20/20	0.007/0.007	0.15/0.15		
<i>E. coli</i>	>20/20	20/20	>20/20	20/20	20/20	20/20	0.01/0.01	0.15/0.15		
<i>P. aeruginosa</i>	20/20	>20/20	>20/20	20/20	20/20	20/20	0.06/0.06	0.63/0.63		
<i>S. enterica</i>	>20/20	20/20	20/20	10/20	20/20	20/20	0.007/0.007	0.15/0.15		
<i>Y. enterocolitica</i>	>20/20	5/20	5/20	2.5/20	10/20	1.25/20	0.007/0.007	0.15/0.15		
Gram-positive bacteria										
<i>B. cereus</i>	>20/20	>10/20	>10/20	10/20	>20/20	>20/20	0.007/0.007	nt/nt		
<i>L. monocytogenes</i>	>20/20	>10/20	>10/20	20/20	20/20	20/20	0.007/0.007	0.15/0.15		
<i>S. aureus</i>	>20/20	20/20	20/20	10/20	10/20	20/20	0.007/0.007	0.15/0.15		
Antifungal activity										
Ketoconazole										
<i>A. brasiliensis</i>	10/20	10/20	10/20	10/20	10/20	10/20	0.06/0.125	—		
<i>A. fumigatus</i>	2.5/20	2.5/5	2.5/5	2.5/20	2.5/5	5/20	0.5/1	—		

nt: not tested. Results are mean  $\pm$  standard deviation ( $n = 3$ ). MIC: minimum inhibitory concentration; MBC: minimal bactericidal concentration. MBC: minimal fungicidal concentration. WF: Wheat flour. GC: Ground chia seed. DGC: defatted ground chia seed. CF: carob flour. WS: White sugar. CS: Coconut sugar.

**Table 4** Antifungal activity (MIC/MBC, mg mL<sup>-1</sup>) of extracts obtained from novel biscuits formulated with ground chia seeds (GC), defatted ground chia seeds (DGC), carob flour (CF) and coconut sugar (CS) as compared with those from traditional ones (control: formulated with wheat flour and white sugar)

	Ground chia seed			Defatted ground chia seed			Carob flour			CS 100% CS100	Carob flour + coconut sugar 100%			Positive control Ketoconazole	
	Control	GC5	GC10	GC15	DGC5	DGC10	DGC15	CF1	CF5	CF10	CF1-CS100	CF5-CS100	CF10-CS100		
<i>A. brasiliensis</i>	10/20	10/20	10/20	10/20	10/20	10/20	5/10	5/10	10/20	10/20	10/20	10/20	10/20	0.06/0.125	
<i>A. fumigatus</i>	1.25/2.5	2.5/5	5/10	2.5/5	2.5/5	2.5/5	2.5/5	2.5/5	5/20	5/20	5/20	5/20	10/20	2.5/20	0.5/1

Results are mean  $\pm$  standard deviation ( $n = 3$ ). MIC: minimum inhibitory concentration; MBC: minimal bactericidal concentration.

coconut sugar being particularly noteworthy for its inhibition of the growth of *Y. enterocolitica* by 1.25 mg mL<sup>-1</sup>, followed by carob flour at 2.5 mg mL<sup>-1</sup> and chia seeds at 5 mg mL<sup>-1</sup>. Similar results were observed in antifungal activity for all the ingredients against *A. brasiliensis*. However, chia seeds and white sugar exhibited the highest fungicidal effect on *A. fumigatus*. This finding suggests a positive impact, especially considering the abundance of this toxicogenic microorganism in cereal crops, particularly in maize, wheat and barley.<sup>37</sup> The antimicrobial activity may be linked to the nutritional composition of both ingredients and biscuits, where phenolic compounds could have had a relevant role. These compounds have the capability to interact with lipids and proteins in the cell membrane, making it not to be semipermeable and causing the leakage of ions and other cellular components. Consequently, this disrupts the metabolism within bacterial cells, ultimately leading to cell death.<sup>38</sup> In this context, several studies have demonstrated that compounds like gallic and protocatechuic acids possess inhibitory effect of *Y. enterocolitica* growth, suggesting their potential application as food preservatives to extend the shelf life of foods.<sup>39,40</sup> In a similar way, ferulic acid has been reported to have antifungal action.<sup>41</sup>

In a recent revision concerning plants utilized as antimicrobials, it has been elucidated that *Salvia* species are notably abundant in various bioactive compounds, including terpene derivatives, essential oils, phenolic compounds, flavonoids, and tannins. These compounds have been closely associated with a range of bioactive properties, including antimicrobial activities, ascribed to *Salvia* species.<sup>42</sup> In this sense, previous research by Kobus-Cisowska *et al.*<sup>43</sup> reported the antimicrobial activity of ethanolic extracts from chia seeds. Consistent with our findings, ground chia seeds have shown antibacterial potential against *L. monocytogenes*, *P. aeruginosa*, and *E. coli*. This activity is primarily attributed to the high content of chlorogenic, ferulic, and protocatechuic acids, with greater extraction efficiency achieved when the seeds are ground. Similarly, antimicrobial properties have been previously described in carob samples.<sup>44</sup> Recently, Djebari *et al.*<sup>45</sup> evaluated the biological activities of extracts obtained from different edible parts of carob tree, including pulp and gum. Their study demonstrated that these extracts could inhibit the growth of pathogenic bacteria at concentrations of 20 mg mL<sup>-1</sup>, and completely halt bacterial growth at higher concentrations ( $>50$  mg mL<sup>-1</sup>). The authors concluded that extracts from carob pulp and gum show promise as alternatives to syn-



thetic additives in the medicinal industry. In this context, they may serve as potential antioxidant agents and preservatives that combat bacterial contamination. In the case of carob, the antimicrobial effect can be mainly attributed to the high content of phenolic acids, gallotannins, and flavonoids.<sup>46</sup> While there is limited information in the literature regarding the antimicrobial activity of sugar samples, certain studies have demonstrated that other coconut-derived products, such as coconut water<sup>47</sup> and virgin coconut oil,<sup>48</sup> are capable of inhibiting the growth of microorganisms such as *Salmonella typhimurium* and *Staphylococcus aureus*.

None of the novel formulations exhibited antibacterial activity against the tested microorganisms as compared with wheat-based biscuits, with the exception of biscuits CF10-CS100, which inhibited the growth of *B. cereus* at concentration of 20 mg mL<sup>-1</sup> (data not shown). However, all biscuits displayed antifungal activity (Table 4). Among them, CF10-CS100 highlighted for its inhibition against *A. brasiliensis* followed by the biscuit containing 15% defatted chia seeds (DGC15). In contrast, the control sample exhibited the highest inhibition against *A. fumigatus*, which proved to be the most sensitivity to all the extracts. Based on the literature, it is anticipated that the activity against microorganisms would diminish or even vanish with heat treatment. Xu *et al.*<sup>49</sup> noted significant reductions in the fungistatic activity of flaxseed when exposed to high temperatures. Similar decreases in antimicrobial properties have been documented in other heat-treated products, including honey.<sup>50</sup> At this point, it is worth considering the possible potential antimicrobial effect of Maillard reaction compounds generated during the heat treatment. In this regard, Diaz-Morales *et al.*<sup>51</sup> reported the antimicrobial properties of melanoidins isolated from various bakery products, including bread and biscuits, suggesting their capacity to enhance food shelf-life and safety. These authors also noted that the antimicrobial properties of melanoidins can vary, influenced by both the microorganism species and the type of melanoidin. This variability could explain the differences observed in the present study for each biscuit, since the development of the products of the Maillard reaction and consequently its activity could depend on the composition of the ingredients and the amount incorporated to the formulations. Previous studies conducted by our research group has indicated that incorporating defatted flour into biscuit formulations led to a substantial rise in Maillard reaction products, including acrylamide and furan compounds.<sup>52</sup> Furthermore, research by Turfani *et al.*<sup>13</sup> highlighted an increase in the browning of wheat bread enriched with carob flour. This was attributed not only to the color of the added flour but also to a higher occurrence of the Maillard reaction during baking, a consequence of elevated lysine content in carob as compared with wheat.

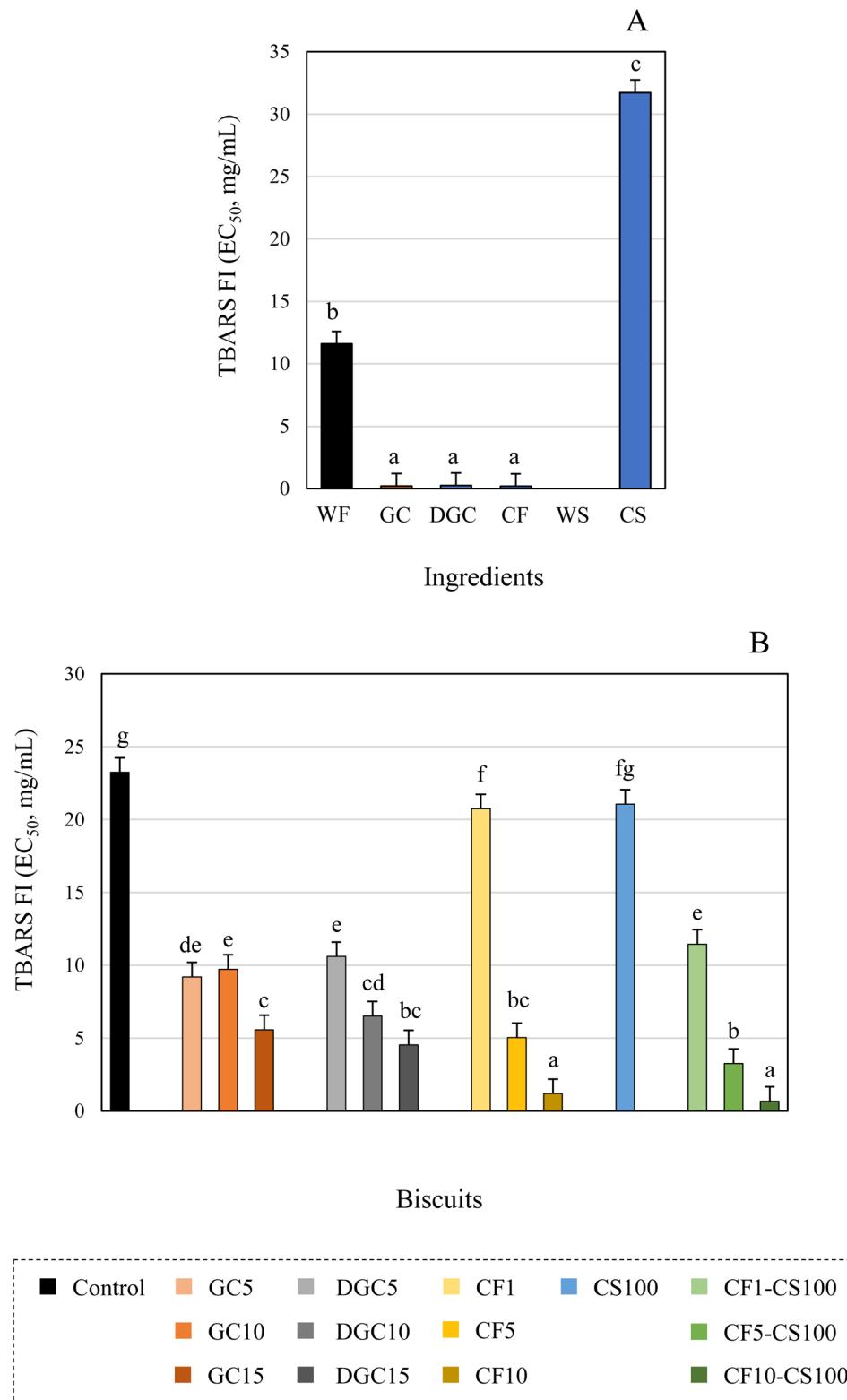
**3.2.2. Antioxidant, anti-inflammatory and cytotoxic activity.** The results of the antioxidant activity of the extracts of both ingredients and biscuit formulations are presented in Fig. 2 and Table 5. Fig. 2 displays the results obtained in the TBARS assay, where higher antioxidant activities are character-

ized by lower EC<sub>50</sub> values. Porcine brain cells were selected due to their high content in polyunsaturated fatty acids. Carob flour and chia seeds exhibited a high capacity to inhibit the formation of TBARS, with EC<sub>50</sub> values ranging from 0.18 mg mL<sup>-1</sup> to 0.20–0.25 mg mL<sup>-1</sup>, respectively. Higher values were detected in coconut sugar (31.72 mg mL<sup>-1</sup>), while white sugar did not present any activity. The addition of these ingredients to the biscuit formulations promoted greater antioxidant activity compared to the control biscuit extracts, reaching EC<sub>50</sub> values up to 5.57 and 4.54 mg mL<sup>-1</sup> when 15% ground chia seeds and defatted ground chia seeds were incorporated in the biscuits, respectively. Concentrations were even lower when samples were formulated with 10% carob flour (1.19 mg mL<sup>-1</sup>), decreasing up to 0.66 mg mL<sup>-1</sup> when white sugar was replaced by coconut sugar.

The high antioxidant capacity of chia seeds has been reported by other authors, mainly associated with the high content of polyphenolic compounds and bioactive peptides.<sup>53,54</sup> Recently, Mas *et al.*<sup>54</sup> reported the ability of chia polyphenols to reduce lipid peroxidation. Coelho *et al.*<sup>53</sup> described that protein hydrolysates from defatted chia seeds have potential *in vitro* and *in vivo* antioxidant capacity and can effectively inhibit lipid oxidation in food models. In agreement with affirmations of Mas *et al.*,<sup>8</sup> the supplementation of biscuits with chia seeds could be recommended to improve antioxidant properties of foods even when heat treated. These authors reported that the addition of 10% defatted chia seeds in cookies increased the polyphenol content and the *in vitro* antioxidant capacity of the formulations. Carob flour has also been noted to enhance the antioxidant capacity of various bakery products. Several studies, have demonstrated that the antioxidant power, measured by FRAP (Ferric Reducing Antioxidant Power) was notably elevated in bread made from blends of wheat flour and carob flours, especially unrefined carob flours at substitutions of 10% or more. Similarly, assays like ABTS (2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl) and HPS (Hydrogen peroxide scavenging) have indicated an increased antioxidant capacity in muffins supplemented with carob powder.<sup>13</sup> This enhancement was linked to a greater contribution of total phenolic compounds and total flavonoids content in the baked goods.<sup>15</sup> In addition to the composition of the innovative ingredients, it is essential to consider the contribution of Maillard reaction compounds generated during baking to the enhancement of the antioxidant capacity in the biscuit samples, as previously reported in other bakery products.<sup>18</sup>

In contrast to the antioxidant activity observed in the TBARS assay, for all the ingredients and biscuit formulations, only chia seeds exhibited cellular antioxidant, anti-inflammatory and cytotoxic activity (Table 5). The highest concentration tested (2 mg mL<sup>-1</sup>) inhibited the oxidation reaction generated in the RAW macrophages by 20% in the extracts from ground chia seed and by 62% in those prepared from defatted ground chia seeds. Regarding the anti-inflammatory activity, the concentration of extract required to inhibit 50% NO production





**Fig. 2** Antioxidant activity of extracts obtained from ingredients (A) (WF: wheat flour, GC: ground chia, DGC: defatted ground chia, CS: coconut sugar) and novel biscuit formulations (B) (TBARS assay). Different letters in ingredients or formulations indicate significant differences between samples ( $p < 0.05$ ).

**Table 5** Antioxidant, anti-inflammatory and cytotoxic activities of extracts obtained from ground chia seeds and defatted ground chia seeds

	Ground chia seed	Defatted ground chia seed
Cellular antioxidant activity (oxidation inhibition % at 2 mg mL <sup>-1</sup> )		
RAW 264.7	20%	62%
Anti-inflammatory activity (IC <sub>50</sub> µg mL <sup>-1</sup> )		
RAW 264.7	18.9 ± 1.7	>400
Cytotoxic activity (GI <sub>50</sub> µg mL <sup>-1</sup> )		
AGS	202.1 ± 4.2a	191.8 ± 6.7a
Caco-2	130.4 ± 11.9a	382.4 ± 8.2b
MCF-7	220.7 ± 6.9a	323.4 ± 2.2b
NCI-H460	298.3 ± 10.0b	175.5 ± 14.3a
Cellular antioxidant activity: quercetin: 95 ± 5% oxidation inhibition at 0.3 µg mL <sup>-1</sup> . Anti-inflammatory activity: IC <sub>50</sub> values for dexamethasone: 6.3 ± 0.4 µg mL <sup>-1</sup> (RAW 264.7). Cytotoxic activity: IC <sub>50</sub> values for ellipticine: 1.23 ± 0.03 µg mL <sup>-1</sup> (AGS), 1.21 ± 0.02 µg mL <sup>-1</sup> (Caco-2), 1.02 ± 0.02 µg mL <sup>-1</sup> (MCF-7), 1.01 ± 0.01 µg mL <sup>-1</sup> (NCI-H460). Different letters in each row correspond to significant differences among extracts (p < 0.05).		

was from 18.9 µg mL<sup>-1</sup> in ground chia seeds whereas defatted samples did not present activity at the maximum concentration tested (> 0.4 mg mL<sup>-1</sup>). The anti-inflammatory effect of chia seed could be due to the presence of lipids in this matrix and particularly to the high content of polyunsaturated fatty acids, which have been previously associated with anti-inflammatory activity by acting as competitor substrates for the inhibition oxidation of arachidonic acid.<sup>55</sup> High levels of polyunsaturated fatty acids in oils obtained from sea bass and bream head oils have been associated with the inhibition of NO in LPS-stimulated macrophage cells.<sup>30</sup> Regarding the cytotoxic activity, the colon cancer cells (Caco-2) and the breast cancer cells (MFC-7) exhibited significantly higher susceptibility to the ground chia seeds (GI<sub>50</sub> = 130.4 µg mL<sup>-1</sup> and 220.7 µg mL<sup>-1</sup>, respectively) as compared with defatted ground chia seeds at the tested concentrations (GI<sub>50</sub> = 382.4 µg mL<sup>-1</sup> and 323.4 µg mL<sup>-1</sup>, respectively). In contrast, defatted samples inhibited to a significant greater extent the proliferation of lung cancer cells (NCI-H460) (GI<sub>50</sub> = 175.5 µg mL<sup>-1</sup> vs. 298.3 µg mL<sup>-1</sup> in ground chia seeds), whereas similar growth inhibition effects of stomach cancer cell lines (AGS) were observed for both samples (GI<sub>50</sub> range: 191.8–202.1 µg mL<sup>-1</sup>). Again, the fat content of the seeds could be considered as responsible for this activity, since previous works have revealed the antiproliferative effect of omega-3 fatty acids against colon and lung cancer cells.<sup>56,57</sup> Together with the fat content, other components of the seeds such as peptides could also be involved in cellular inhibition, which would explain the activity in the defatted ground chia seed.<sup>58</sup>

## 4. Conclusions

The partial replacement of wheat flour with ground chia seeds and carob flour and the total replacement of white sugar with

coconut sugar in biscuit formulations enhanced the protein, mineral and particularly the fiber content. Additionally, the incorporation of chia seeds resulted in elevated levels of omega-3 fatty acids, which are essential for the human health. The new ingredients exhibited antibacterial and anti-fungal activity, particularly against *Yersinia enterocolitica* and *Aspergillus fumigatus*, although the final biscuits only displayed antifungal properties. The remarkable ability of chia seeds and carob flour to inhibit the formation of TBARS remained in the biscuits and the antioxidant capacity was even increased when white sugar was replaced by coconut sugar. Chia seeds were the only ingredient displaying cellular antioxidant, anti-inflammatory, and cytotoxic activities, which were not observed in the biscuit formulations. The results of this study emphasize the significant health benefits associated with the incorporation of these innovative ingredients into traditional biscuits. Moreover, they underscore the potential applications of these ingredients in the food industry, particularly when integrated into new cereal-based formulations.

## Author contributions

M. M., F. J. M., L. B. and E. P. developed the study design, methodology and research. M. M., C. C., T. C. S. P. P. and R. C. C. performed the data collection. M. M. carried out the main analysis with supervision from T. C. S. P. P., R. C. C. and E. P. M. M. wrote the first and final drafts of the manuscript. F. J. M., C. C., T. C. S. P. P., R. C. C., L. B. and E. P. critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

## Conflicts of 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.

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