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Defatted chia flour as functional ingredient in sweet cookies. How do Processing, simulated gastrointestinal digestion and colonic fermentation affect its antioxidant properties?

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ABSTRACT

The aim of this work was to improve the antioxidant quality of cookies using defatted chia flour (DCF), which is a by-|product of the food industry. We prepared cookies containing DFC (5, 10 and 20%), and evaluated the technological and sensory qualities of cookies. Additionally, we verified the effects of processing and simulated gastrointestinal digestion on polyphenols content. The addition of DFC did not affect the technological quality of cookies, with the exception of color. Furthermore, cookies supplemented with 10% DFC were sensorial preferred over the others. The addition of DFC increased the polyphenol content and the *in vitro* antioxidant capacity of cookies. Besides, the simulated gastrointestinal digestion suggested that 73% of total polyphenols could be absorbed in the intestine, showing an antioxidant effect greater than expected, also showing prebiotic effects. Supplementation of cookies with 10% DFC could be recommended to improve antioxidant quality without reducing the technological or sensorial properties.

1. Introduction

In addition to their basic nutritional functions, there are certain food components with demonstrated beneficial effects on human health. Polyphenols are a group of compounds well known by their antioxidant properties. This antioxidant capacity depends not only on the amount of polyphenols but also on their particular chemical structure. The polyphenol profile of food can be modified along its elaboration, as a consequence of mechanical and chemical processes, and also during the gastrointestinal digestion. Bioactive compounds must resist these processes to be able to be absorbed, enter the blood and reach the target organism/tissue (Caicedo-Lopez et al., 2019; Lingua, Wunderlin, & Baroni, 2018). Moreover, some polyphenols are attached/bounded to other food components (e.g. proteins) and need to be released from the food matrix. In this context, the evaluation of the potential antioxidant effect of foods and their ingredients requires a deep knowledge about the polyphenols profile, including information on their bioaccessibility and bioavailability as well.

Chia seeds (*Salvia hispanica* L.) are widely known by their high content of ω-3 fatty acids, with demonstrated beneficial effects on the cardiovascular system (Capitani, Spotorno, Nolasco, & Tomás, 2012). For this reason, chia oil is extracted from the seeds at industrial scale, and the non- fatty portion (defatted chia flour, DFC) is discarded. Considering that DFC is rich in phenolic compounds, fibers, etc., (Aranibar et al., 2018), we are interested in promoting its use as a functional ingredient in different foods, generating supplemented products which are rich in natural antioxidants, in addition to decreasing industrial waste.

Short dough biscuits are usually made with wheat flour, sugar and fat. These ingredients are responsible for the distinctive characteristics of this product. Any modification in the original formulation can affect the dough, generating technological issues that can have a negative

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effect on the texture and sensorial parameters. Therefore, it is important to carefully evaluate the incorporation of new ingredients, looking to preserve both the quality and acceptability of cookies (Blanco Canalis, León, & Ribotta, 2017).

Different studies have been carried out using chia seeds, evaluating their composition, and the effects on different food matrices (Giaretta, Lima, & Carpes, 2018; Mesías, Holgado, Márquez-Ruiz, & Morales, 2016; Verdú, Barat, & Grau, 2017). However, to our knowledge, there are no studies addressing the elaboration of a food supplemented with DCF, considering, at the same time, the evaluation of its technological, sensorial and antioxidant characteristics, in addition to the study of bioavailability and bioaccessibility of polyphenols as representatives of improved antioxidant capacity.

We hypothesized that the addition of DCF should improve the antioxidant capacity of cookies by enhancing the amount and variety of polyphenols in the composition, but this improved antioxidant ability could be modified from raw materials to processed cookies, including changes throughout their digestion.

In sum, the main goal of this work was to improve the antioxidant quality of sweet cookies by supplementation with DCF, preserving both technological and sensory qualities, and verifying changes in the phenolic profile as a consequence of both the elaboration and digestion processes.

2. Material and methods

2.1. Chemicals and reagents

Ultra-pure water (< 18 MΩ·cm, < 5 μg L^{-1} TOC) was obtained from a purification system Arium 61316-RO plus Arium 611 UV (Sartorius, Germany). Methanol (HPLC grade) and formic acid (puriss. p.a. for mass spectroscopy) were provided by J. T. Baker (State of Mexico, Mexico) and Merck (California, USA), respectively.

Commercial standards of ferulic acid and caffeic acid were obtained from Extrasynthese (Genay, France), catechin, myricetin, tryptophan, rosmarinic acid, quinic acid and isoquercitrin, from Sigma-Aldrich (Steinheim, Germany), and quercetin and kaempferol, from Fluka (Dorset, U.K.). Filters (0.45 μm, HVLP04700) were obtained from Millipore (São Paulo, Brazil). ABTS (2,2′-azino-bis-(3-thylbenzothiazolne-6-sulfonic acid) diammonium salt), DPPH (1,1-diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-tripyridyl-S-triazine), Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), Folin-Ciocalteu reagent, pepsin (P-7000, from porcine stomach mucosa), pancreatin (P-1750, from porcine pancreas) and bile extract (B-8631, from porcine) were purchased from Sigma-Aldrich (Buenos Aires, Argentina). SnakeSkin dialysis bags with a molecular weight cut-off of 10 kDa and a width of 22 mm, and Hypersep SPE 500 mg/2.8 mL C18 cartridges were obtained from ThermoFisher SCIENTIFIC. Anaerobic atmosphere generation bags were purchased from Mitsubishi Gas Chemical (Tokyo, Japan). Different culture media were used: De Man, Rogosa and Sharpe Agar (MRS, Biokar diagnostics), Reinforced Clostridial Agar (RCA, Biokar diagnostics), Eosin Methylene Blue (EMB, Britania) Agar, and Bile Esculin Agar (BEA, Britania). All other reagents were of analytical grade.

2.2. Samples

2.2.1. Defatted chia flour (DCF) preparation

Chia seeds were obtained from commercial plantations in the province of Salta (Argentina). The oil extraction was carried out by cold pressing in a single step (Komet screw press model CA 59 G, IBG Monforts, Germany). The moisture content of the seeds was adjusted to 0.11 g/g dry basis, the pressing temperature was 30 °C, the screw speed was 20 rpm, and the restriction die was 6 mm (Bodoira, Penci, Ribotta, & Martínez, 2017). The remaining deoiled portion was milled in a laboratory grinder (Tecnodalvo, Santa Fe, Argentina) obtaining the DCF.

2.2.2. Preparation of cookies

Four cookies formulations were prepared containing 0, 5, 10 and 20% of DCF in replacement of wheat flour. The formulation without DFC was named as Control Cookie (CC). The other three formulations were identified as CFC5, CFC10 and CFC20, respectively, where CFC means Chia Flour Cookie. The ingredients used per cookie batch were wheat flour (45 g), caster sugar (27 g), vegetable shortening (20.20 g), powdered skimmed milk (2.25 g), $NaHCO_3$ (0.50 g), NaCl (0.42 g), and water (4 mL). Depending on the formulation, some wheat flour was replaced with the corresponding amount of DCF (2.25; 4.50 and 9.00 g, respectively).

Short dough was manually stretched with a rolling pin and sheeted to a 0.8 cm thickness. Afterwards, it was cut using a metallic cutter of 4.5 cm of diameter. The cookies were placed on greaseproof paper, and distributed separately on an aluminum tray. Then, they were placed in the center of a forced convection oven (Pauna, Argentina) equipped with a temperature controller, and baked for 11 min at 180 °C (Blanco Canalis et al., 2017). The recipe yielded 6 cookies.

After baking, cookies were cooled down to room temperature and 4 cookies were selected to perform the technological and sensory analyses. Those used for the polyphenol analysis, antioxidant activity and gastrointestinal digestion model were frozen and stored at −80 °C until further analysis.

2.3. Technological quality of cookies

To determine the technological quality of the new formulations, different analyses were carried out. Hardness was measured as the strength required to produce the total break of the cookie with an INSTRON Texturometer (Model 3342, Norwood, MA, USA) equipped with a 500 N cell. The base gap of the two support beams was adjusted to 36 mm, the travel distance of the blade was 35 mm, and speed was 0.5 mm/s. Fractal dimension and the area of cracking were measured with an image of the surface of the cookies using the image-processing program FIJI. Color was determined using a colorimeter (CM spectrophotometer KONICA MINOLTA Sensing, INC), which defines each color from three coordinates in the CIE Lab color space: L* (luminosity), a* (red-green) and b* (yellow-blue). Finally, the term cookie factor was defined as the ratio between the width and height of four cookies selected at random. This was used as a measure of quality; higher values were correlated to a better quality (Barrera, Pérez, Ribotta, & León, 2007; Blanco Canalis et al., 2017).

2.4. Sensory evaluation

Cookie samples were evaluated by 36 healthy adults (semi-trained assessors) the day after cooking. All formulations, including the control cookie (CC), were identified with arbitrary three-digit numbers, and were presented to each assessor at the same time in a completely randomized order. Samples were evaluated with a descriptive analysis, qualifying surface appearance, aroma, sweet taste, crunchiness, hardness and chewiness. Drinking water was provided for palate cleansing between each sample. Discontinuous bipolar 7-point structured scales were used where zero represented the lowest intensity, and 7 represented the highest intensity of a particular attribute. Sensory instructions as well as the arrangement provided to assessors for sensory attributes evaluation are shown in the Supplementary Material (Table 1 and Appendix 1). Afterwards, a preference analysis was carried out; assessors were asked to sort all the formulations in order of preference (being #1 the most accepted sample, and # 5 the least accepted one).

2.5. In vitro digestion and colonic fermentation

A complete *in vitro* digestion model was simulated in four stages: the digestive process in the mouth, stomach (gastric), small intestine and large intestine, including colonic fermentation (Gil-Sánchez et al.,

Table 1
Technological analysis of new cookie formulations. Different letters indicate significant differences ($p < 0.05$) between formulations for the same parameter.

	Cookie Factor	Hardness (N)	Fractal dimension (D)	Area of cracking (%)	L	a*	b*
CC	3.6 ± 0.22^a	97 ± 11^b	1.05 ± 0.03^c	0.78 ± 0.30^c	80.57 ± 2.32^a	3.14 ± 0.71^b	26.61 ± 2.90^a
CFC5	3.67 ± 0.10^a	120 ± 13^a	1.23 ± 0.09^b	2.19 ± 0.84^b	72.71 ± 2.80^b	3.92 ± 0.81^a	24.85 ± 3.13^b
CFC10	3.62 ± 0.18^a	127 ± 11^a	1.27 ± 0.07^b	2.37 ± 0.19^b	69.55 ± 2.29^c	3.58 ± 0.83^a	22.64 ± 2.82^c
CFC20	3.83 ± 0.06^a	93 ± 11^b	1.41 ± 0.09^a	4.37 ± 0.28^a	63.22 ± 2.32^d	3.93 ± 1.06^a	20.57 ± 2.30^d

2017; Lingua et al., 2018; Minekus et al., 2014; Pham et al., 2017).

Briefly, two grams of cookie were homogenized in presence of freshly collected human saliva (2 mL) for 30 s at 24,000 rpm in an Ultra-Turrax T18 blender (Ika-Labortechnik, Germany) to simulate mastication. The pH was immediately adjusted to 2 with 6 M HCl, to stop the action of amylase, and to condition the medium to further continue with the gastric digestion.

After that, the mixture was incubated at 37 °C for 2 h with constant agitation at 60 oscillations per minute with the aggregate of pepsine from porcine gastric mucosa in 0.1 M HCl (2000 U/mL final concentration) to simulate the gastric digestion.

The next step was to mimic the digestion and absorption in the small intestine. For this purpose, a solution containing pancreatin and bile salts in 0.1 M $NaHCO_3$, pH = 7.5 (100 U trypsin activity/mL and 10 mM of final concentration, respectively) was mixed with the homogenate from the previous step. This mixture was placed inside a dialysis bag, which allowed simulating the passive absorption of the polyphenolic compounds through the membrane of the small intestine. The dialysis bag was then immersed in 0.1 M $NaHCO_3$ (pH = 7.5) and incubated in darkness, with agitation at 40 osc/min for 3 h at 37 °C. The fraction that passed through the dialysis membrane (identified as Small Intestine Dialyzable, SID), was then separated. This portion represented the fraction available for absorption into the circulatory system by passive diffusion in the small intestine.

The remaining solution inside the bag, the non-dialysable fraction, was used to continue the last step of colonic fermentation and passive absorption in the large intestine. A mice model for colonic fermentation was used, considering that most reports on the biological effects of chia and rosmarinic acid were also performed in mice (Carnier et al., 2017; Gonçalves et al., 2019). Thus, we can compare our results with those from the literature. A sterile medium consisting of peptone (2 g), yeast extract (2 g), NaCl (0.1 g), K_2HPO_4 (0.04 g), KH_2PO_4 (0.04 g), $NaHCO_3$ (2 g), $MgSO_4{\cdot}7H_2O$ (0.01 g), $CaCl_2{\cdot}6H2O$ (0.01 g) and Tween 80 (2 mL) was added into the dialysis bag. All these amounts are expressed per liter of final solution. Additionally, mice fecal slurry was added into the dialysis bag at a final concentration of 10^6 CFU/mL. The fecal slurry was prepared by mixing 1 g of fecal sample (balb c mice with standard diet) in 10 mL of sterile phosphate-buffered saline. Afterwards, the dialysis bag was immersed in 0.1 M $NaHCO_3$ (pH = 7.5) and incubated in an anaerobic chamber in the dark, with agitation at 40 osc/min for 24 h at 37 °C. The fraction that passed through the dialysis membrane (Large Intestine Dialyzable, LID) represented the fraction available for absorption into the circulatory system by passive diffusion in the large intestine. The non-dialyzable fraction (ND) represented the remaining material in the colon tract, which would be finally excreted.

This assay was performed in triplicate with CFC10 and CC samples. Additionally, three blank samples (without cookies) were processed and analyzed to discard the influence of the digestion reagents on phenolic compounds and antioxidant capacity.

2.5.1. Sample preparation for analysis

All fractions from the *in vitro* digestion and colonic fermentation were purified and concentrated with a SPE C-18 cartridge (Di Paola-Naranjo, Sánchez-Sánchez, González-Paramás, & Rivas-Gonzalo, 2004).

The cartridge, previously pre-conditioned with methanol and ultra pure water, was rinsed with the samples and washed with 0.1% of formic acid in ultra pure water. Afterwards, the polyphenols were recovered using methanol with 0.1% formic acid and stored at −80 °C until further analysis.

The ND fractions were previously centrifuged at 10.000 rpm for 10 min. The supernatant was purified and concentrated with the SPE C-18 cartridge, and the pellet was used to determine the prebiotic capacity of DCF.

2.6. Antioxidant properties analysis

2.6.1. Polyphenol extraction

Before the extraction, cookies were ground and defatted with hexane. Ground cookies were extracted in a proportion of 1 g to 5 mL of *n*-hexane shaking for 1 h at room temperature. Then, the supernatant was removed and vacuum filtered. This procedure was repeated three more times.

Subsequently, one gram of sample (DCF or CFC) was extracted with 5 mL of a mixture of Methanol/Water (5:5) and sonicated for 15 min in an ultrasonic bath (Cleanson, Argentina). Then the extracts were centrifuged (Gelec, Argentina) for 10 min at 800 × *g*, and the supernatants were collected. This process was repeated three more times and all supernatants were combined, filtered and stored at −80 °C until HPLC-MS analysis and antioxidant properties measurement.

2.6.2. Determination of Total Polyphenol Content (TPC)

Total polyphenol content (TPC) was determined using the Folin Ciocalteu method (Singleton & Rossi, 1965). Briefly, the absorbance of properly diluted samples with the addition of Folin-Ciocalteu reagent and an aqueous solution of sodium carbonate 20% was read at 750 nm. TPC was calculated using a calibration curve constructed with gallic acid. Results were expressed as micrograms of polyphenols (equivalent to gallic acid) per g of sample (whether DCF or CFC). All samples were analyzed in triplicate. Blank samples (containing only the reagents) were used to discount the absorbance due to solvents and reagents.

2.6.3. Polyphenol profile

The phenolic profile of samples was determined by HPLC-DAD-MS/MS following the method by Lingua et al. (2018) using an Agilent Series 1200 LC System (Agilent, Santa Clara, CA, USA), coupled to a DAD detector (Agilent Series 1200) in tandem with an ESI source, connected to a mass spectrometer (Micro-QTOF II; Bruker Daltonics, Billerica, MA, USA). Polyphenols in samples were identified according to their retention times, exact mass, UV/Vis spectra, MS and MS/MS spectra, and compared with authentic standards when available. When authentic standards were not available, a tentative identification was performed using UV–VIS, exact MS and MS/MS, considering reports from compounds in the literature. Quantification of polyphenols was based on external calibration curves from available phenolic standards, using the mass peak areas obtained from the extracted ion chromatograms, at concentrations between 0.025 and 100 ppm. A quantification mix was prepared in different concentrations within that range. The mix contained the following standards: ferulic, caffeic, rosmarinic and quinic acid, isoquercitrin, quercetin, kaempferol and myricetin. When the corresponding standards were not available, the quantification was performed using an external standard with a structure similar to the tentative compound. Limits of detection (LOD) and quantification

(LOQ) of the method used to quantify the phenolic compounds were experimentally calculated from the calibration curves. Precision of the method was evaluated by calculating the coefficients of variation (CV) from, at least, nine determinations covering the specified range for the procedure. LOQ ranged from 0.04 to 0.54 ppm, and LOD, from 0.01 to 0.16 ppm. All samples were analyzed in triplicate including the analysis of blanks to verify the effects of matrix on the MS detection.

2.6.4. In vitro antioxidant activity

The antioxidant capacity (AC) was measured by FRAP (Ferric Reducing Antioxidant Power) and TEAC (Trolox Equivalent Antioxidant Capacity / Radical Scavenging) methods (Benzie & Strain, 1996; Re et al., 1999). In brief, 100 μL of the properly diluted sample were mixed with the corresponding reagent and measured at 593 nm and 734 nm, respectively. In both cases, the results were obtained from a calibration curve made using Trolox. Results were expressed in μg of Trolox equivalents per g of DCF or CFC. All samples were analyzed in triplicate. Blank samples (containing reagents) were used for each sample type (cookies or digestion samples) to discount the absorbance due to solvents and reagents.

2.7. Prebiotic effect

Before and after colonic digestion, *Enterobacteriaceae, Lactobacillus, Enterococcus, Clostridium* and *Bifidobacterium* were counted using Eosin methylene blue agar (EMB), de Man, Rogosa and Sharpe (MRS), Bile Esculin Agar (BEA) and MRS supplemented with 5% v/v with propionic acid, respectively. Serial dilutions of the samples were performed and seeded in the corresponding agar plates. The incubation was performed in an anaerobic jar using anaerobic atmosphere generation bags from Mitsubishi Gas Chemical.

A quantitative equation was used to help with the analysis of prebiotic effect of fermentation (Paesani, Salvucci, Moiraghi, Fernandez Canigia, & Pérez, 2018). This prebiotic index (PI) takes into account the UFC of beneficial bacteria (*Lactobacillus* and *Bifidobacterium*) and non-beneficial groups (*Enterobacteriaceae* and *Clostridium*). The equation was PI = (Lact/Total) − (*Ent*/Total) + (Bif/Total) − (Clost/Total), where Lact, *Ent*, Bif and Clost are CFU of *Lactobacillus*, *Enterobacteriaceae*, *Bifidobacterium* and *Clostridium*, respectively.

2.8. Statistical analysis

For the statistical analysis the software INFOSTAT (Di Rienzo, Casanoves, Balzarini, Gonzalez, Tablada, & Robledo, 2011) was used. Data are expressed as mean ± SD. Analysis of variance (ANOVA) was performed with each variable to evaluate differences between results. In the case of significance ($p < 0.05$), a LSD Fisher comparison test was performed to reveal paired differences between the means.

3. Results and discussion

3.1. Total polyphenol content and antioxidant capacity of DCF and CFC

Considering that chia seeds are rich in polyphenolic compounds, the addition of DFC is expected to cause an increase in the antioxidant properties of a wheat flour formulation.

Folin-Ciocalteu analysis confirmed this assumption showing an increased TPC of supplemented cookies when compared to the control cookie (CC) (Fig. 1). TPC values for the different formulations were close to those theoretically expected from the TPC of DCF (6424 μg/g). Also, the amount of polyphenols was increased along with the quantity of DFC added to the cookies.

Antioxidant activity was evaluated by two different methods: radical scavenging (TEAC) and reducing power (FRAP). According to the chemical structure of compounds, they will react diversely in the *in vitro* assays due to the different mechanisms involved (hydrogen atom transfer, single electron transfer, reducing power, and metal chelation, among others).

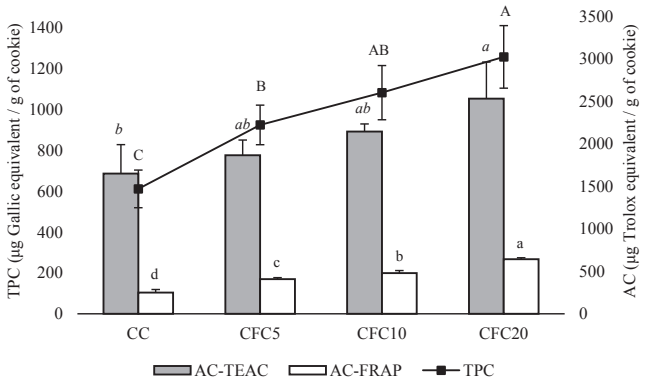

Fig. 1. Total Polyphenol Content (TPC) and Antioxidant Capacity (AC) of Control and Chia supplemented Cookie. Different letters indicate significant differences ($p < 0.05$) between different formulations for the same method.

For both methods, an increment associated with the proportion of DCF added was also observed, but the values obtained were barely below those theoretically expected (80–90%). Interestingly, the tendency of an AC increased by a higher content of chia is less noticeable for the TEAC assay, indicating that the action mechanism of these polyphenols could be mainly related to their reducing power (FRAP). Previous reports highlighted the lack of correlation between the total polyphenolic content and different antioxidant activity assays, suggesting that the AC is highly correlated not only with the content but also with the composition of phenolic profile (Baroni et al., 2018).

Mesías et al., 2016 and Costantini et al., 2014, who added chia flour in different percentages to biscuits and bread, respectively, found similar results for TPC and AC.

3.2. Technological quality of cookies

Physical properties are an important aspect for consumers when choosing a product. Both manufacturing conditions and raw materials are factors affecting these properties (Nakov, Brandolini, Ivanova, Dimov, & Stamatovska, 2018). Short dough cookies are expected to present reduced thickness but large diameter; with a tender but crunchy texture and a uniform surface cracking (Blanco Canalis et al., 2017). As a good quality parameter, CC is an accepted formulation. Results of the technological analysis are summarized in Table 1.

In particular, high cookie factor is known as a good characteristic for cookies (Barrera et al., 2007). The addition of DCF did not modify the width and height of cookies, suggesting that dough rheology was not affected (Blanco Canalis, Valentinuzzi, Acosta, León, & Ribotta, 2018), obtaining products of acceptable quality in all formulations.

Another important physical parameter to consider in the quality evaluation of cookies is hardness, measured as the resistance to breaking by the three point bend method. CFC5 and CFC10 hardness was slightly higher than control ($p < 0.05$). It is well known that hardness of cookies is related to water-starch-protein interactions and also to the presence of fiber, so the higher values observed can be associated with the higher protein and fiber content of DFC (Aranibar et al., 2018; Giuberti et al., 2018). However, CFC20 showed similar hardness to control cookies, possible indicating that the highest level of addition produced a dilution effect of flour components affecting texture. Interestingly, Mesías et al. (2016) determined lower hardness values in cookies supplemented with chia flour. This difference with respect to our results is probably because they used whole chia flour, with oil content.

In addition, another important technological parameter is a high degree and uniform cracking pattern on the cookie surface. These

phenomena occur during baking as a consequence of dough dehydration and sucrose recrystallization in the biscuit surface (Manley, 2000). Higher area fraction and fractal dimension mean higher degree of cracking and surface roughness (Blanco Canalis et al., 2018). All formulations with DCF showed significant differences in this parameter compared to control cookie, although there were no differences between CFC5 and CFC10. Supplemented formulations showed more cracked and complex surfaces, being CFC20 the one with the greatest values.

Finally, an important physical characteristic of a food product is color. Color development occurs during baking and is mainly due to the degradation of sugars, known as the Maillard reaction, and caramelization (Nakov et al., 2018). In our experiments, changes in color could be attributed also to the presence of natural pigments in DCF. The addition of DCF decreased the whiteness (L*) of cookies and the b* parameter, while the a* parameter was increased, showing significant differences with CC (Fig. 1 of Supplementary Material). These parameters indicate that cookies became darker with increased proportions of DCF, in accordance with the results reported by Mesías et al. (2016).

In summary, although the addition of DCF modified some quality parameters, the overall technological quality of supplemented cookies was similar to the control, indicating minor effects of DCF on the final product.

3.3. Sensory analysis of cookies

One of the main issues in food formulation when including novel ingredients is the possible adverse effect on the consumers' acceptance of the product. To assess this aspect, thirty-six semi-trained adults evaluated the different cookies formulations using a discontinuous bipolar 7-point structured scale, where zero represented the lowest intensity, and 7 represented the highest intensity of a particular attribute. Table 2 shows the results of the sensory evaluation.

Surface appearance was increased with the DCF addition, showing significant differences with CC and between all the CFC formulations. These results are in agreement with the values obtained for fractal dimension, area of cracking and color in the technological analysis (Section 3.2.). Aroma perception showed significant differences with CC, 10 and 20% DCF cookies, whereas none of the formulations showed changes in the perception of sweet taste. Crunchiness did not show a clear tendency due to high data dispersion (SD ≈ 30%). With respect to hardness, even though the technological study showed differences among samples, the assessors did not recognize significant differences between formulations. Finally, no significant differences were found in chewiness among samples.

With respect to the preference analysis, the assessors concluded that CFC10 was the preferred formulation, followed by CFC5, CFC20 and CC. This shows that the supplementation with DCF improves the sensory acceptance of these cookies. Coelho and Salas-Mellado (2015) found similar results regarding the acceptance of bread supplemented with 7.8% of chia flour. On the other hand, Martins, Pinho, and Ferreira (2017) established that in sensory analysis, addition of byproducts functional ingredients to biscuit formulations usually decreases scores related with aroma, flavor and texture. Our results show that DCF is a good alternative as a functional ingredient, since it improves the antioxidant characteristics of cookies, maintaining an acceptable technological quality and a good sensory acceptance.

Taking into account the results obtained from the technological and sensory analyses, where CFC10 was preferred over the other formulations, this sample was selected to study the effect of processing and digestion on chia polyphenols.

3.4. Changes in polyphenol profile caused by processing

In order to evaluate changes in phenolic profile, we first determined the composition of DCF. Twenty-five compounds were tentatively identified in DCF (Table 1 of Supplementary Information). Quantitative data is shown in Table 3. Thirteen of the identified compounds were hydroxycinnamic acids, structurally related to caffeic acid. The most important ones were salviaflaside, rosmarinic acid and fertaric acid, adding up to almost 60% of all polyphenols. Additionally, ten flavonoids including quercetin, kaempferol, myricetin and some glycosylated derivatives were found. Compounds identified and quantified in this work are in agreement with those previously reported in chia seeds extracts (Rahman, Costa de Camargo, & Shahidi, 2017). Finally, one organic acid (quinic acid) and one aminoacid (tryptophan) were found in DFC. Even though the last two compounds are not strictly polyphenols, they are included in this study because they have demonstrated antioxidant effects (Nayak & Buttar, 2016; Pero, Lund, & Leanderson, 2009).

Of the compounds present in DCF, only 11 were found in CFC10 after processing: six hydroxycinnamic acids (danshensu, caftaric acid, fertaric acid, salviaflaside isomer I and II and rosmarinic acid isomer II), three flavonoids (quercetin dihexoside, Kaempferol dihexoside and quercetin hexoside isomer I), and only one organic acid (quinic acid) and aminoacid (tryptophan). None of these compounds were identified in CC except for a considerable amount of tryptophan (9.74 μg/g), which is a known component of wheat flour (Podio, Baroni, & Wunderlin, 2017). Regarding the quantification of polyphenols in CFC10, and considering their content in the DCF and the proportion added (Table 3), approximately 100% of polyphenols were recovered, but with a distinct phenolic profile.

Taking into account each compound individually, quinic acid, danshensu and kaempferol dihexoside showed a recovery higher than 100% (but their concentration was too low to affect the total concentration of polyphenols), while other polyphenols such as caftaric, fertaric and rosmarinic acid II revealed a recovery lower than expected. Finally, for the rest of the compounds, the percentages of recovery were close to 100%.

These changes may be due to chemical and physical modifications during processing. For example, it is known that polyphenols in plants could be linked to the cell wall, but they could be released by heating or kneading, which accounts for the recovery percentages above 100%. On the other hand, this type of compounds could interact with the components of the new matrix (in this case, cookies) such as lipids and carbohydrates, reducing their availability, and making their extraction more difficult. In addition, some polyphenols could also be degraded by heating during cooking, or by oxidation during kneading, thus explaining lower percentages of recovery after processing (Jakobek, 2015; Kardum & Glibetic, 2018).

Briefly, the amount of total polyphenols remains as expected, but the initial proportion of each compound is modified, which may explain

Table 2
Sensory analysis of new cookie formulations. Different letters indicate significant differences ($p < 0.05$) between formulations for the same attribute.

	Surface Appearance	Aroma	Sweet Taste	Crunchiness	Hardness	Chewiness
GC	0.19 ± 0.54^{d}	3.41 ± 0.29^{b}	3.46 ± 0.75^{a}	3.32 ± 0.91^{b}	3.64 ± 0.48^{a}	3.61 ± 0.69^{a}
GRC5	2.60 ± 0.95^{c}	3.20 ± 0.65^{b}	3.06 ± 0.72^{a}	4.30 ± 1.26^{a}	4.16 ± 1.24^{a}	3.50 ± 0.90^{a}
GRC10	3.46 ± 1.08^{b}	3.68 ± 0.89^{a}	3.29 ± 0.79^{a}	3.32 ± 1.39^{b}	3.65 ± 1.15^{a}	3.80 ± 1.02^{a}
GRC 20	5.29 ± 1.74^{a}	3.68 ± 1.00^{a}	3.45 ± 0.76^{a}	5.26 ± 1.02^{a}	4.04 ± 1.18^{a}	4.04 ± 1.11^{a}

Table 3
Quantification of polyphenols in DCF, CFC10 and the digested and fermented extracts. The media and standard deviation are informed in ppm (μg/g).

N°	Rt [min]	Compound	Concentration [ppm]				
			DCF	CFC10	SID	LID	ND
1	7,8	Quinic acid	58.3 ± 10.63	11.93 ± 3.88	0.29 ± 0.09 (2.45)	< LOD	< LOD
2	11.8	Danshesu	17.25 ± 5.33	2.79 ± 0.51	< LOD	< LOD	< LOD
3	12.2	Caftaric acid	166.43 ± 25.99	3.30 ± 0.67	1.15 ± 0.49 (34.85)	0.56 ± 0.05 (16.99)	0.77 ± 0.10 (23.21)
4	12.5	Tryptophan	87.63 ± 22.66	25.80 ± 14.02	1.29 ± 0.32 (5.00)	0.96 ± 0.30 (3.73)	5.22 ± 1.22 (20.23)
5	13	Caffeic acid hexoside	27.72 ± 5.09	< LOD	< LOD	< LOD	< LOD
6	13	Salvianolic acid I/H	28.69 ± 10.56	< LOD	< LOD	< LOD	< LOD
7	13.2	Myricetin dihexoside	4.00 ± 1.33	< LOD	< LOD	< LOD	< LOD
8	13.3	Fertaric acid	374.57 ± 38.83	12.10 ± 2.48	2.41 ± 0.16 (19.92)	4.13 ± 0.66 (34.13)	2.46 ± 0.92 (20.32)
9	14.2	Quercetin dihexoside	16.62 ± 6.62	1.245 ± 0.60	< LOD	< LOD	< LOD
10	14.2	Salvianolic acid E/B/L	150.53 ± 22.06	< LOD	< LOD	5.18 ± 0.46	2.72 ± 0.79
11	14.6	Caffeic acid	11.08 ± 3.18	< LOD	0.61 ± 0.20	0.48 ± 0.14	0.82 ± 0.40
12	16	Kaempferol dihexoside	0.43 ± 0.22	0.14 ± 0.01	< LOD	< LOD	< LOD
13	16.1	Myricetin hexoside	15.62 ± 4.40	< LOD	< LOD	< LOD	< LOD
14	17.3	Salviaflaside isomer I	1137.82 ± 79.71	58.01 ± 12.69	10.27 ± (17.70)	9.68 ± 0.92 (16.68)	1.71 ± 0.53 (2.96)
15	18.2	Salviaflaside Isomer II	226.15 ± 40.10	13.59 ± 1.91	7.19 ± 2.18 (52.90)	6.70 ± 1.28 (49.31)	6.94 ± 1.66 (51.05)
16	19	Quercetin hexoside Isomer I	12.69 ± 4.31	0.58 ± 0.18	< LOD	0.19 ± 0.04 (32.89)	< LOD
17	19.1	Rosmarinic acid Isomer I	42.83 ± 9.64	< LOD	< LOD	< LOD	< LOD
18	19.6	Rosmarinic acid Isomer II	740.08 ± 39.21	14.51 ± 7.73	6.88 ± 2.14 (47.43)	47.21 ± 1.12 (325.44)	12.89 ± 2.69 (88.86)
19	20.4	Quercetin hexoside Isomer II	0.86 ± 0.13	< LOD	< LOD	< LOD	< LOD
20	20.8	Quercetin deoxyhexoside	1.19 ± 0.66	< LOD	< LOD	< LOD	< LOD
21	21.2	Myricetin	20.61 ± 5.62	< LOD	< LOD	< LOD	< LOD
22	21.6	Salvianolic acid C	39.29 ± 14.26	< LOD	< LOD	< LOD	< LOD
23	22.5	Methyl rosmarinate	26.65 ± 11.13	< LOD	< LOD	< LOD	< LOD
24	24.1	Quercetin	40.61 ± 14.56	< LOD	< LOD	< LOD	0.20 ± 0.09
25	26.9	Kaempferol	1.25 ± 0.93	< LOD	< LOD	< LOD	< LOD
Total			3250.45	144.00	30.10 (20.90)	75.51 (52.44)	33.74 (24.13)

SID: Small Intestine Dialyzable; LID: Large Intestine Dialyzable; ND: Non-dialyzable fraction. < LOD, below limit of detection. Recovery percentage of the compound in parenthesis. Recovery percentages in the digested and fermented fractions were calculated in relation to CFC10. Compound 1 was quantified using quinic acid as reference compound; compounds 2, 3, 5 and 11 were quantified using caffeic acid; compounds 9, 16, 19 and 20 using isoquercitrin; compound 4 using tryptophan; compounds 6, 10, 14, 15, 17, 18, 22 and 23 using rosmarinic acid; compounds 7, 13 and 21 using myricetin; compound 8 using ferulic acid; compounds 12 and 25 using kaempferol; and compound 24 using quercetin.

the changes observed in the antioxidant capacity (Section 3.1). Abdel-Aal and Rabalski (2013) obtained similar results, finding that some polyphenols increased and others decreased after cooking. They also demonstrated that, depending on the bakery product, bread or cookie, the modifications suffered are different. Conversely, Kaderides, Mourtzinos, and Goula (2019) observed a loss in most of pomegranate peel polyphenols when added to cookies, confirming the need to microencapsulate the compounds to protect them from baking. Altogether, these results indicate that there is not a direct connection between heat treatment and changes on phenolic profile, suggesting that every modification should be carefully studied for each particular case.

3.5. Changes in polyphenol profile and antioxidant properties caused by the simulated digestion and colonic fermentation

To study the bioaccessibility of polyphenols present in CFC10 as well as the changes in their antioxidant properties, the three biologically important fractions resulting from the *in vitro* digestion were analyzed: SID (Small Intestine Dialyzable fraction), LID (Large Intestine Dialyzable fraction), and ND (Non Dialyzable fraction). The same analysis was performed for CC and blank samples of digestion.

The dialyzable fractions correspond to the bioaccessible polyphenols, which would be absorbed by passive diffusion to perform their antioxidant effect. Although this model does not consider active absorption, it is a novel and relatively simple way to approximate results to real *in vivo* digestion. Table 3 summarizes the concentration of each polyphenol as well as their recovery in the different fractions. The AC of all fractions is shown in Fig. 2.

3.5.1. Polyphenol profile

From the eleven compounds observed in CFC10, only seven were detected in SID (mainly phenolic acids, and quinic acid and tryptophan in a much smaller quantity). All of them showed recovery percentages below 50%. The major compounds in this fraction were the same as those found for CFC10 (rosmarinic acid and salviaflaside). Similar results were reported by Pellegrini et al. (2018) for chia seeds, furthermore they found that defatted chia seeds had a better bioaccessibility and bioavailability than non-defatted chia seeds. Pesic et al. (2019) the reported that food matrix affects the bioaccessibility and bioavailability of polyphenols. On the other hand, caffeic acid, a compound from chia flour, but not detected in cookies, was detected after digestion, showing that the action of pH and enzymes made it more available as previously reported by different authors (Caicedo-Lopez et al., 2019; Pesic et al., 2019). With respect to the LID fraction, after fermentation of the remaining portion from the previous stage by colonic bacteria, ten phenolic compounds were found. Salvianolic acid E/B/L was detected in this sample, a compound present in chia flour, but not detected in cookies, which means that it became accessible after fermentation. A decrease in the recovery of caftaric acid and tryptophan was observed, and quinic acid was not detected. The most interesting aspect about this fraction is the recovered amount of rosmarinic acid, which was even higher than in CFC10. This result demonstrates that colonic fermentation could cause the release of phenolic compounds increasing their accessibility (Jakobek, 2015). Similar results were obtained by Attri, Sharma, Raigond, and Goel (2018) who reported an increase in the concentration of certain compounds in the first 24 h of fermentation, which then decrease. However, it is difficult to make comparisons with our results since our model includes a dialysis step to simulate passive absorption. This fraction showed the highest concentration of phenolic compounds.

Finally, nine polyphenols were found in the ND fraction, most of them being the same as those detected in LID, with the additional presence of quercetin in a very low concentration (compound not detected in the previous fraction, nor in CFC10), and with the absence of

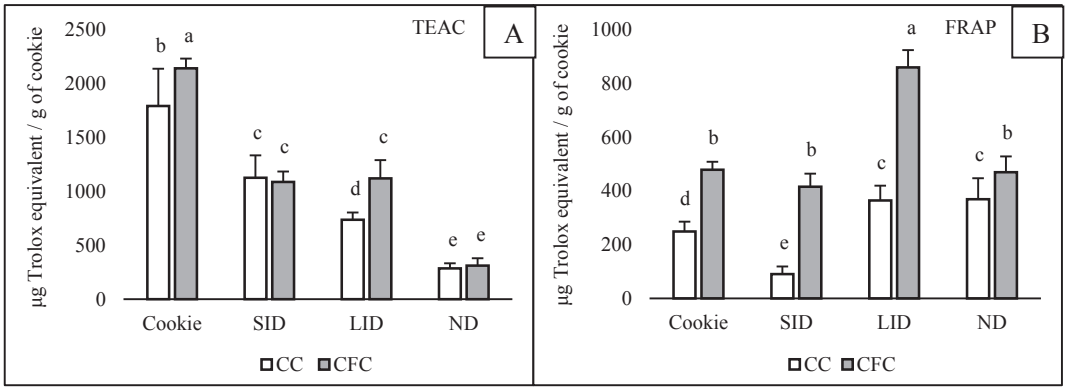

Fig. 2. Antioxidant Capacity (AC) of CFC, CC and the digested and fermented extracts. Different letters indicate significant differences ($p < 0.05$) between different fractions for the same method. A- TEAC method, radical scavenging activity. B- FRAP method, reducing power.

quercetin hexoside isomer I. Probably, this finding is explained by the hydrolysis of quercetin hexoside to quercetin. A greater proportion of tryptophan could also be observed, together with a significant decrease in fertaric acid, salviaflaside isomer I, and rosmarinic acid isomer II.

As it could be observed, there are significant changes in the polyphenol profile and in the proportion of each compound. The sum of individual compounds (ΣPolyphenols) showed recoveries of 20.85, 52.31 and 23.37% for SID, LID and ND, respectively. In total, 96.53% of polyphenols quantified in CFC10 were detected in these fractions, with 73.15% corresponding to the dialyzed portion which represents the passively absorbed compounds in the intestine and to the effectively bioactive fraction.

3.5.2. Antioxidant properties

Polyphenols can exert their antioxidant activity through different ways in the organism. Although *in vitro* techniques do not show the real biological effect, they are a good approach to the antioxidant effect of these extracts.

Considering the radical scavenging capacity (TEAC), SID fraction strongly decreases its effect compared to the CFC10 extract, being statistically equivalent to the same fraction of CC (Fig. 2). This is probably because the technique is not sensitive enough to differentiate the antioxidant activity of the small quantity of polyphenols with respect to CC, due to the low recovery of compounds in this stage. Although Pellegrini et al. (2018) obtained different results, with increased TEAC values for defatted chia seeds after small intestine digestion, these differences probably arise from the varying digestion models used, which did not include a dialysis step. However, after colonic fermentation (LID), the radical scavenging capacity showed differences with respect to the same CC fraction; which may be due to the high recovery of polyphenolic compounds in this fraction, shown in Section 3.5.1. Regarding the ND portion, the same effect as in LID was observed.

Finally, the reducing capacity (FRAP) showed significant differences in all fractions. The antioxidant activity of the SID fraction increased the difference between CFC and CC, with respect to the undigested cookies, in contrast to the results of the TEAC method. This may be because the antioxidant activity does not only depend on the amount of polyphenols, but also on their structure, suggesting that the mechanism of action of these polyphenols is mainly through their reduction power (Uranga, Podio, Wunderlin, & Santiago, 2016). The other fractions (LID and ND) showed the same trend as that observed for TEAC, with a considerable increase in the dialyzed portion after fermentation, and decreasing for the non-dialyzable portion. Similar results were obtained by Attri et al. (2018) for antioxidant activity after colonic fermentation.

In summary, the antioxidant capacity and the polyphenols profile were significantly modified by the gastrointestinal digestion, following different trends depending on the mechanism of action, but showing an increase in CFC with respect to the control cookies due to colonic fermentation. These results suggest that a certain amount of polyphenols from CFC would be absorbed by the organism and exert their antioxidant effect, with an important role of the intestinal microbiota contributing to the absorption.

3.6. Prebiotic capacity

Prebiotics are compounds that resist gastrointestinal digestion and can be fermented by microorganisms from the large intestine, selectively modifying the growth or activity of certain bacteria. The modulation of the microbiota can result in a regulatory effect not only locally but also at immunological or neuroendocrine levels, generating a significant impact on health. There are evidences suggesting the role of polyphenols as a stimulating factor for growth and development of beneficial gut microbiota (Mahajan, Attri, Mehta, Udayabanu, & Goel, 2018). Therefore, it is interesting to evaluate the prebiotic effect of polyphenols that were not dialyzed in the simulated absorption model.

After colonic fermentation, the growth of different bacteria genera was affected. Fig. 3 summarizes the results obtained. The sample called *inoculum* refers to samples before colonic fermentation (t = 0 h), while the rest of the samples corresponds to 24 h of fermentation.

Firstly, *Enterococcus*, *Bifidobacteria* and *Lactobacillus* showed the same trend, growing after colonic fermentation and even more in presence of CFC10; although there were only statistically significant differences in the case of *Lactobacillus*. *Clostridium* showed similar growth after 24 h of fermentation in all samples. In the case of *Enterobacteriaceae*, both CC and CFC10 decrease their growth to the point of not showing significant differences with respect to the inoculum before fermentation. Although there is no difference between CC and CFC10, there was a tendency that CFC10 would further decrease its growth. Similar results have been observed in batch colonic fermentations with polyphenolic-rich extracts from different sources as reviewed by Mahajan et al. (2018). Attri et al. (2018) observed an increment in the proportion of lactic acid bacteria and bifidobacteria when fermentation was made with sea buckthorn berries. Similar trends for increase in Lactobacillus sp. and Bifidobacterium sp. were observed using cocoa extract (Tzounis et al., 2011), blueberry extract (Molan, Lila, Mawson, & De, 2009) and grape seed extract (Cueva et al., 2013). Polyphenols are in general biotransformed by gut microbiota, at first by deglycosylation, followed by a breakdown of flavonoids into smaller metabolites. Furthermore, these metabolites may modulate the growth of bacteria in the gut (Attri et al., 2018).

In summary, CFC10 would help to increase the growth of beneficial bacteria from the colonic microbiota and decrease the possibility of growth of non-beneficial groups.

In reference to the prebiotic index, CFC10 and CC showed statistically significant differences with values of 6.26 and 2.51, respectively. This result demonstrates that the DFC added to the formulation

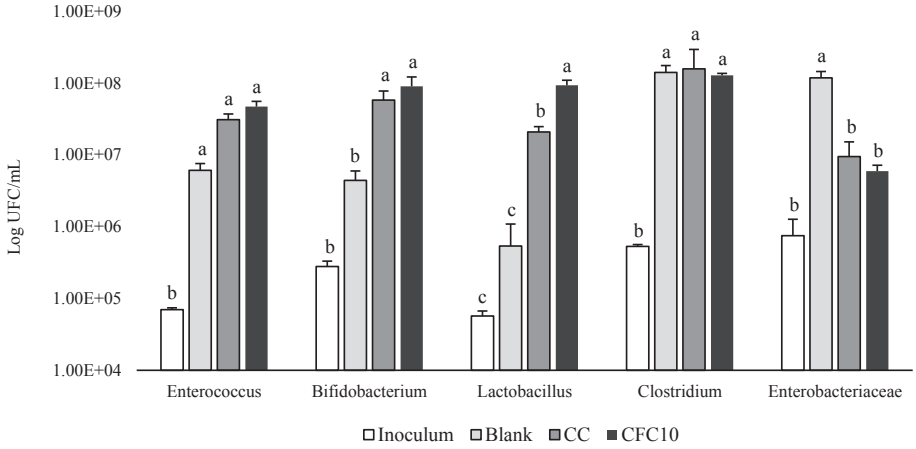

Fig. 3. Bacterial count after colonic fermentation (t = 24 h) of Blank, CC and CFC10. The Slurry sample (Inoculum) was measured before fermentation (t = 0 h). Different letters indicate significant differences ($p < 0.05$) between different samples for the same bacteria genus.

succeeded in producing an increased growth of beneficial bacteria genera and supported the effect of CFC10 as a prebiotic.

4. Conclusions

Our current results demonstrate the advantages of using DCF as a functional ingredient to improve the antioxidant properties of sweet cookies. DCF-supplemented cookies retain technological quality and good sensorial attributes, since CFC10 was preferred over other formulations. The addition of DCF increased the antioxidant capacity (TEAC and FRAP assays) and the polyphenol content (Folin-Ciocalteu and HPLC-MS/MS) of cookies. After processing, the quantitative polyphenol profile was modified, although rosmarinic acid, salviaflaside and fertaric acid remain as the most abundant components in processed DCF-cookies. The gastrointestinal digestion also affected the quantitative phenolic profile, with only few polyphenols released from the food matrix, and absorbed by passive diffusion in the small intestine. On the other hand, colonic fermentation produced a greater release of polyphenols, in addition to a significant increase in the antioxidant capacity. Besides, DFC polyphenols also showed prebiotic effects, which represent an additional beneficial effect of the consumption of DCF-supplemented cookies.

Thus, this work reports a novel antecedent on the benefits of using DCF as a functional ingredient in the formulation of foods, enhancing the antioxidant capacity without lowering technological and sensorial qualities. Further research, using *in vivo* analyses with cell cultures or animal models would be required to get a deeper understanding of the real antioxidant effect of these seeds, and their beneficial effects on human health.

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CRediT authorship contribution statement

Agustín Lucini Mas: Investigation, Formal analysis, Writing - original draft. **Federico Iván Brigante:** Investigation, Formal analysis. **Emiliano Salvucci:** Methodology, Formal analysis. **Natalia Belén Pigni:** Methodology, Formal analysis, Writing - review & editing. **Marcela Lilian Martinez:** Methodology, Formal analysis. **Pablo Ribotta:** Methodology, Writing - review & editing. **Daniel Alberto Wunderlin:** Conceptualization, Resources, Project administration. **María Verónica Baroni:** Conceptualization, Resources, Visualization, Supervision, 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126279.

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