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Article

Gastrointestinal Digestion Impact on the Phenolic Composition and Bioactivity of Biscuits with Tannat Grape Pomace

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Abstract: The search for natural sources of bioactive compounds with health-promoting properties has intensified in recent years. Among these, Tannat grape pomace (TGP), a primary byproduct of winemaking, stands out for its high phenolic content, although its bioactivity can be affected during gastrointestinal digestion. This study aimed to evaluate the impact of *in vitro* digestion on the antioxidant (ABTS, ORAC-FL, intracellular ROS inhibition), antidiabetic (α -glucosidase inhibition), anti-obesity (lipase inhibition) and anti-inflammatory (NO inhibition) properties of five sugar-free biscuits formulated with varying percentages of TGP and sucralose. No significant differences were observed in the bioaccessible fractions (BFs, representing compounds potentially released in the small intestine) between control biscuits and those enriched with TGP, suggesting limited release of phenolics at this stage. Conversely, colonic fractions (CFs, simulating the material reaching the colon) from biscuits with higher TGP content exhibited greater bioactivities. HPLC-DAD-MS analysis of the CF from the biscuit containing 20% TGP and 4% sucralose revealed a high content of procyanidin trimers, indicating the persistence of these specific phenolic compounds after *in vitro* digestion. These findings suggest that TGP-enriched biscuits may deliver health benefits at the colonic level and support their potential application in the formulation of functional foods.

Keywords: Bioaccessibility; Bioactivity; Biscuits; *In vitro* Digestion; Phenolic Compounds; Tannat grape pomace

1. Introduction

The increasing consumer demand for natural and sustainable ingredients has significantly intensified the scientific interest in food byproducts as promising sources of bioactive compounds with potential health benefits [1]. As a result, emerging research areas are focusing on studying the health effects of winery byproducts, particularly their role in improving several disorders related to oxidative stress and inflammation, which are risk factors for the development of chronic diseases [2]. Grape pomace, the main solid byproduct of winemaking is composed primarily of seeds and peels and is a rich and natural source of health-promoting compounds, including dietary fiber and phenolic compounds that remain in the pomace after juice extraction [3,4]. Among grape varieties, Tannat grape pomace (TGP) has been identified as a rich source of dietary fiber (64%) as well as phenolic compounds, being the main ones malvidin-3-O-(6'-p-coumaroyl) glucoside, (+)-catechin, malvidin-3-

O-glucoside and (-)-epicatechin [5]. These compounds found in TGP offer significant health benefits, such as antioxidant, prebiotic, anti-inflammatory, antiproliferative, antiobesity and antidiabetic properties [4].

Despite TGP having such bioactive properties, the beneficial effects on health depend on the extent to which these compounds are released during gastrointestinal digestion (bioaccessibility) and subsequently absorbed into systemic circulation (bioavailability) [6,7]. It is estimated that only 5–10% of dietary polyphenols are absorbed in the small intestine, primarily those in aglycone forms [8]. Most polyphenols reach the colon, where they interact with the gut microbiota or are excreted [6]. Moreover, when phenolic compounds are incorporated into food products, they can interact with macromolecules present in the food matrix, which may affect their bioaccessibility, bioavailability and consequently the impact they could have on health [5,9]. For all these reasons, investigating the interaction between polyphenols and the intestinal microenvironment is essential for clarifying the mechanisms underlying the health benefits of polyphenol-rich foods [7,10].

In this context, the present study aimed to evaluate the functional properties of sugar-free biscuits enriched with different concentrations of TGP and sucralose, following *in vitro* gastrointestinal digestion simulation. The research focused on evaluating the antioxidant, antidiabetic, anti-obesity and anti-inflammatory properties of the fractions generated from *in vitro* digestion of the biscuit formulations: the bioaccessible fraction, representing the portion potentially available for absorption in the small intestine and the colonic fraction, corresponding to the material that reaches the colon for potential microbial metabolism. Furthermore, phenolic compounds present in the colonic fraction of the most bioactive formulation were identified using HPLC-DAD-MS to explore potential associations between chemical composition and biological activity.

2. Results and Discussion

2.1. Phenolic Compounds Profile in the Colonic Fraction (CF)

Phenolic compounds identified in the CF of TGP and the biscuit containing 20% TGP and 4% sucralose (20% 4%) are presented in Table 1. No phenolic compounds were detected in the CF of the control biscuit with 4% sucralose (data not shown). The 20% 4% biscuit formulation was selected for analysis due to its high antioxidant activity as measured by ABTS and ORAC-FL (Figure 2).

In the CF of the 20% 4% biscuit, the predominant compound was procyanidin trimer (Rt 9.10 min) followed by malvidin-3-O-(6'-p-coumaroyl)glucoside. In contrast, the CF of TGP showed malvidin-3-O-glucoside, malvidin-3-O-(6'-p-coumaroyl)glucoside, procyanidin trimer (Rt 9.0 min) and (+)-catechin as the main compounds, in descending order. Except for the procyanidin trimer (Rt 9.10 min) all identified compounds had higher peak areas in the CF of TGP than in the biscuit CF. This compound was tentatively identified as a procyanidin trimer based on its retention time, UV-Vis absorption spectrum and characteristic mass fragmentation pattern obtained by HPLC-DAD-MS. Previous studies have suggested that polymeric procyanidins can interact with digestive enzymes, which may lead to their precipitation and retention in the insoluble fraction during simulated digestion [11]. A decrease in flavan-3-ols, specifically polymeric procyanidins, has been previously reported in the BF of grape pomace extracts [12] further supporting the idea that these compounds are not efficiently released into the soluble phase and instead remain in the CF. In addition to precipitation, the polymerization of phenolic compounds during digestion due to pH changes is proposed as another possible hypothesis to explain their higher relative content [6]. This predominant presence of procyanidin trimers obtained in the present study could be beneficial for consumer health. Once in the colon, procyanidins and their monomeric units may undergo microbiota metabolism, altering their bioavailability and resulting in the production of low molecular weight phenolic acids with potential health benefits [13,14]. Regarding other grape byproducts, some phenolic compounds (syringic and cinnamic acids, ϵ -viniferin, myricetin and naringenin) from grapevine bunch stem and cane from Malbec grape cultivar have been found to be highly bioaccessible [15]. However, procyanidins (monomers, dimers and trimers) bioaccessibility was

very low or completely missed. Accordingly, cereal-based food matrix with extracts from other grape byproducts has shown proanthocyanidins and total flavonoids low bioaccessibility [16]. Similarly, the bioaccessibility studies of heat-treated skim goat-milk powder fortified with grape-pomace-seed extract showed low recovery of flavan-3-ols and phenolic acids [17].

A comparative analysis between the biscuit before [5] and after *in vitro* digestion (Table 1) revealed a 56% increase in the total peak area in the CF of the digested biscuit compared to the undigested sample. This increase in the total peak area may be partially explained by the release of phenolic compounds during digestion, likely due to the disruption of interactions with macromolecules in the biscuit matrix [18]. These interactions may hinder full compound recovery during extraction from undigested samples, resulting in an underestimation of phenolic content prior to digestion [12,19]. Several phenolic compounds originally present in TGP and in the undigested biscuit were not detected in their CFs after digestion. Interestingly, many of the compounds absent from the CF of both TGP and the biscuit, such as cis-caftaric acid, protocatechuic acid, trans-caftaric acid, p-coumaroyl hexose, two procyanidin trimers (Rt 16.02 and 16.32), procyanidin dimers (B2, B7, and galloylated forms) and flavonol glycosides (myricetin-3-O-galactoside, quercetin-3-O-galactoside, syringetin-3-O-glucoside), were also not detected in the undigested biscuit. This suggests a possible degradation or transformation of these compounds during thermal processing and/or gastrointestinal digestion, where enzymes and pH changes may alter their stability and bioavailability [20,21].

Conversely, some compounds—such as (–)-epicatechin gallate, delphinidin-3-O-glucoside, and cyanidin-3-O-glucoside—were not originally detected in TGP but were identified in its CF, suggesting that they may have been released from the food matrix or formed as transformation products during digestion [12,19].

To our knowledge, this is the first study to identify phenolic compounds in the insoluble fraction of digested biscuits formulated with grape pomace, highlighting the potential of winemaking byproducts as sources of bioactive compounds.

Table 1. Data on identification of phenolic compounds in the Colonic Fraction (CF) of TGP and of the biscuit with 20% of TGP and 4% of sucralose (20% 4%) analyzed by HPLC-DAD-MS. Rt: Retention time.

Compound		CF TGP		CF Biscuit 20%4%	
		Rt (min)	Area	Rt (min)	Area
Chromatogram 280 nm	Phenolic acids	Trans-caftaric acid	6.911 16981		
	Flavan-3-ols	Procyanidin trimer C2	4.62 4624	4.867 1495	
		Procyanidin dimer B1	7.334 9819	7.224 2166	
		Procyanidin dimer B3	7.795 63891	7.768 21267	
		(+)-Catechin	8.335 130356	8.274 41044	
		Procyanidin trimer	9.008 185897	9.109 271845	
		Procyanidin trimer	10.554 99312	10.509 30335	
		Procyanidin dimer B4	11.397 35471	11.299 9612	
		Procyanidin dimer B6	11.952 22016	11.826 3210	
		(–)-Epicatechin	12.442 84897	12.379 28992	
		Galloylated procyanidin dimer	15.364 19076	15.346 14467	
		(–)-Epicatechin gallate	18.389 6723		
	Flavonols	Myricetin-3-O-glucoside	21.816 9007		
		Quercetin-3-O-glucoside	23.388 60556	23.385 4589	
		Quercetin-7-O-neohesperidoside	28.161 26998	28.204 8073	

Chromatogram 520 nm	Anthocyanins	Quercetin (aglycone)	35.086	44860	35.165	15043
		Delphinidin-3-O-glucoside	14.221	6325	14.236	1490
		Cyanidin-3-O-glucoside	16.208	2038		
		Petunidin-3-O-glucoside	17.65	54033	17.709	6960
		Peonidin-3-O-glucoside	19.669	36138	19.771	1733
		Malvidin-3-O-glucoside	20.771	398827	20.925	42507
		Petunidin-3-O-(6'-acetyl)glucoside	26.555	10056	26.61	1621
		Peonidin-3-O-(6'-acetyl)glucoside	28.907	6793		
		Malvidin-3-O-(6'-acetyl)glucoside	29.508	39730	29.555	4603
		Delfinidin-3-O-(6'-p-coumaroyl)glucoside	30.471	26936	30.527	9299
		Malvidin-3-O-(6'-caffeoyl)glucoside	32.356	30092	32.39	3240
		Cyanidin-3-O-(6'-p-coumaroyl)glucoside	32.766	8846	32.812	2113
		Petunidin-3-O-(6'-p-coumaroyl)glucoside	33.448	55245	33.495	17043
		Peonidin-3-O-(6'-p-coumaroyl)glucoside	35.865	37621	35.913	10862
		Malvidin-3-O-(6'-p-coumaroyl)glucoside	36.19	362321	36.236	121228

2.2. Total Phenolic Content (TPC)

The evaluation of total phenolic content (TPC) across the BF_s and CF_s aimed to determine the extent to which phenolic compounds were released or retained following *in vitro* digestion. As shown in Figure 1, the TPC in BF_s of biscuits with and without TGP addition showed no significant differences ($p>0.05$) indicating that phenolic compounds were not effectively released during the small intestinal phase of digestion. In contrast, the TPC of the CF_s differed significantly ($p<0.05$) among formulations, with biscuits containing 20% and 15% TGP exhibiting the highest TPC values. The presence of non-bioaccessible phenolic compounds reaching the colon along with indigestible polysaccharides has been previously reported [22,23]. Although, to our knowledge, there is no available data regarding TPC in the CF of grape pomace, similar behavior has been observed in other agro-industrial byproducts such as avocado peel. It has been reported that a large proportion of phenolic compounds (approximately 66%), including condensed and hydrolyzable tannins, resist gastrointestinal digestion and remain in the non-digestible fraction, allowing them to reach the colon and undergo microbial metabolism [24].

To evaluate the effect of digestion, the TPC of the biscuit containing 20% TGP and 4% sucralose (20% 4%) was compared before [5] and after simulated gastrointestinal digestion (Figure 1). The undigested sample showed a TPC of 1.85 ± 0.04 mg GAE/g. After digestion, the BF exhibited a non-significant increase to 2.60 ± 0.42 mg GAE/g ($p>0.05$), whereas the CF reached a significantly higher value of 8.44 ± 0.62 mg GAE/g ($p<0.05$), indicating a higher concentration of phenolic compounds retained in the CF. The lack of significant change in the BF suggests limited release of phenolic compounds during the upper gastrointestinal phase, consistent with previous findings showing that most dietary phenolics have low absorption rates in the small intestine due to their strong interaction with food matrix components [8,25]. The increase in the CF may reflect their partial release or concentration in the insoluble fraction as a result of digestion.

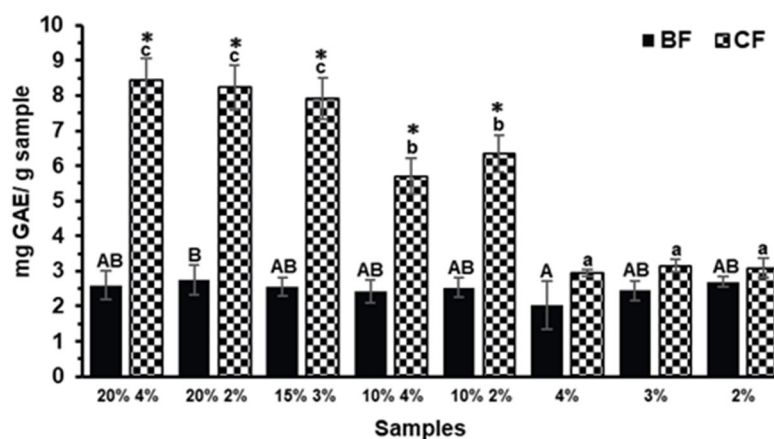


Figure 1. Total Phenol Content (TPC) of the Bioaccessible and Colonic Fractions (BFs and CFs, respectively) of the different Tannat grape pomace (TGP) biscuits (TGP% sucralose%: 20% 4%, 20% 2%, 15% 3%, 10% 4% and 10% 2%) and their control biscuits (sucralose%: 4%, 3%, 2%). Bars denote the mean values and error bars the standard deviation. Different lowercase letters represent significant differences between the CFs of the different biscuit formulations (Tukey test, $p < 0.05$). Different capital letters represent significant differences between the BF of the different biscuit formulations (Tukey test, $p < 0.05$). Asterisks (*) indicate significant differences between BF and CF within the same biscuit formulation (T-test, $p < 0.05$).

2.3. Antioxidant Capacity

Phenolic compounds can be transformed in the digestive tract [26] making it crucial to determine the mechanism of action of antioxidant compounds once they transit through the gastrointestinal tract. Regarding the antioxidant compounds present in the CF (Figure 2) the same trend as the TPC results was observed, with the biscuit formulations containing a higher percentage of TGP (20% and 15%) showing the highest ABTS values (Figure 2a). On the other hand, the compounds in the BF of the biscuits with TGP predominantly exhibited the HAT mechanism of action by ORAC-FL method (Figure 2b), reaching similar values to the ones of CF. The antioxidant capacity in the BF cannot be attributed solely to the presence of phenolic compounds derived from TGP since, as a similar antioxidant capacity was observed for the BF of the biscuits with and without TGP (control biscuits) ($p > 0.05$). During digestion, proteins are hydrolyzed, releasing bioactive peptides [27] which have been shown to possess antioxidant, antidiabetic, antihypertensive, and anti-inflammatory properties [28]. The findings of this study indicate that the antioxidant compounds in the BF after *in vitro* digestion may include bioactive peptides derived from the proteins in the flour and eggs of the biscuits [27].

To the best of our knowledge, there are no previous reports on the antioxidant capacity of the BF and CF after *in vitro* digestion of products with grape pomace, although there are data related to other grape byproducts as well as other polyphenolic sources. Consistent with the findings of this study, the BF of a yogurt with Tannat grape skin showed similar values to the control yogurt (without the byproduct) [29]. However, in the same study the BF of a biscuit with Tannat grape skin showed increased ABTS values when compared to the control biscuit (without the byproduct) [29]. Moreover, cereal-based food matrix enriched with extracts from other grape byproducts have shown to increase the total polyphenol content and to contribute to their antioxidant capacity after *in vitro* digestion [16]. In contrast to the findings of the present study, results reported by Odriozola-Serrano et al. [30] showed that after *in vitro* gastrointestinal digestion of a rosehip extract, the CF exhibited lower antioxidant capacity (measured by DPPH and FRAP) compared to its BF. Conversely, after chemical-enzymatic digestion of avocado peel, no antioxidant capacity was observed in its BF, as measured by DPPH, ABTS, and FRAP. However, antioxidant capacity was observed in the non-digestible fraction, suggesting that phenolic compounds may reach the colon during gastrointestinal digestion [24]. Considering these reports from another plant sources, it is important to highlight the role of the matrix in the release process of phenolic compounds during digestion, making bioaccessibility

studies necessary for each case. In addition, the phenolic composition must be taken into consideration as well because of different stability and chemical transformations during digestion [25].

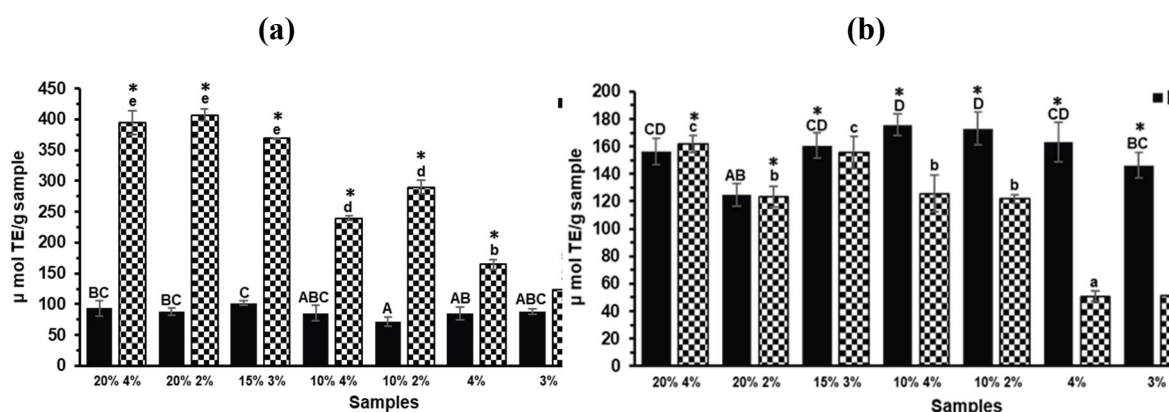


Figure 2. Antioxidant capacity measured by (a) ABTS and (b) ORAC-FL of the Bioaccessible and Colonic Fractions (BFs and CFs, respectively) of the different TGP biscuits (TGP% sucralose%: 20% 4%, 20% 2%, 15% 3%, 10% 4% and 10% 2%) and their control biscuits (sucralose%: 4%, 3%, 2%). Bars denote the mean values and error bars the standard deviation. Different lowercase letters represent significant differences between the CFs of the different biscuit formulations according to Tukey test ($p < 0.05$). Different capital letters represent significant differences between the BF of the different biscuit formulations according to Tukey test ($p < 0.05$). Asterisks (*) indicate significant differences between BF and CF within the same biscuit formulation (T-test, $p < 0.05$).

In addition to determining the mechanisms of action of antioxidant compounds, it is crucial to evaluate their impact in cell lines, as these methods may not accurately reflect the potential effects of antioxidants in living systems [31]. In this context, the BF of both biscuits (same concentration of BF 20% 4% and BF 4%) showed no significant differences ($p > 0.05$) between them in their capacity to reduce intracellular ROS formation (Figure 3). However, both BF significantly reduced ($p < 0.05$) intracellular ROS formation when compared to the positive control (AAPH) and the digestion blank (data not shown), indicating that the observed effect may be exerted by the compounds from the ingredients of the biscuit generated during digestion. The BF may contain bioactive peptides released from the proteins in the biscuit ingredients by the *in vitro* digestion [27], which could interact with cells and trigger antioxidant responses by influencing cellular signaling pathways that regulate the expression of antioxidant enzymes [32]. Conversely, the CF of the 20% 4% biscuit significantly ($p < 0.05$) reduced intracellular ROS formation when compared to the CF of the control biscuit and the positive control (AAPH). This suggests that the compounds present in the CF of the TGP biscuit may play a crucial role in counteracting the effects of oxidative stress in the colon cells. Similar protective effects against intracellular ROS generation were observed in previous studies, where the bioaccessible fraction of yogurt with Tannat grape skin reduced ROS formation in normal human colon fibroblasts (CCD-18Co cells), which could be partially attributed to the phenolic compounds and bioactive peptides released from the yogurt matrix during digestion [29], emphasizing the influence of the food matrix on the bioaccessibility of phenolic compounds. According to Martins et al. [33], polyphenols present in a grape pomace extract obtained from mixed varieties (Syrah and Seibel) can effectively eliminate peroxy radicals in the extracellular compartment under AAPH-induced oxidation, before these can react with the probe in the intracellular compartment. Thus, this could be a possible explanation of the mechanism of action of TGP polyphenols in the reduction of intracellular ROS formation in colon cells. To the best of our knowledge, this is the first study to investigate the antioxidant effect of the insoluble fraction after *in vitro* digestion on normal human colon cells.

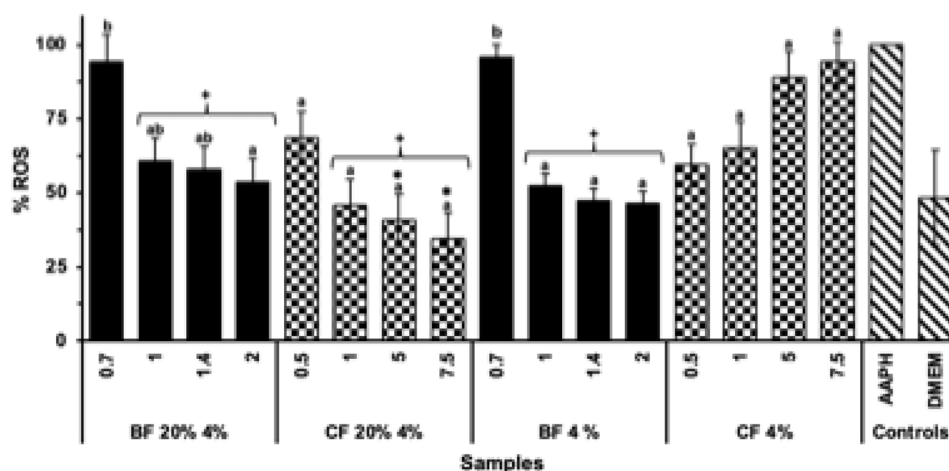


Figure 3. AAPH (1 mM)-induced ROS formation in normal human colon cells (CCD 841 CoN) treated with different concentrations of the Bioaccessible and Colonic Fractions (BF and CF, respectively) of 20% 4% biscuit (TGP% sucralose%) and its control biscuit (4%, sucralose) without TGP. Bars represent the mean values and error bars represent the standard error. Different letters denote significant differences between concentrations (mg/mL) of the same sample (Tukey, $p < 0.05$). Asterisks (*) indicates significant differences between the same concentrations (mg/mL) of both BF or CF samples (T-test, $p < 0.05$). +Indicates significant differences between the sample and the positive control (AAPH) (T-test, $p < 0.05$).

2.4. Antidiabetic and Antiobesity Capacities

The evaluation of the antidiabetic and antiobesity properties of the biscuits after *in vitro* digestion aimed to elucidate the interactions between the compounds present in the digestive fractions (BF and CF) and key gastrointestinal tract enzymes, such as α -glucosidase and pancreatic lipase.

Regarding α -glucosidase inhibition (Figure 4a) no significant differences were observed in the BF across all formulations, including the controls ($p > 0.05$). This suggests that the inhibitory activity in the BF is not primarily driven by polyphenols from TGP but may instead be attributed to compounds released from the biscuit matrix during digestion. For instance, peptides derived from egg white hydrolysates have been reported to inhibit α -glucosidase [34]. In contrast, CFs from TGP biscuits exhibited significantly higher inhibition ($p < 0.05$) than their respective controls, suggesting a contribution from phenolic compounds retained in the colonic fraction. The inhibitory activity of polyphenols is closely related to their chemical structure, which facilitates interaction with enzyme active sites or substrates [35]. Notably, the procyanidin trimer, the predominant compound identified in the CF of the 20% TGP biscuit (Table 1), may play a central role. Proanthocyanidins, due to their polymeric nature and multiple interaction sites, have demonstrated potent inhibitory effects on α -amylase and α -glucosidase [36].

For pancreatic lipase inhibition (Figure 4b), neither the BFs of any formulation nor the CFs of control biscuits exhibited measurable inhibitory activity (data not shown). However, the CFs of formulations containing 20% and 15% TGP showed significant inhibition, indicating that phenolic compounds in the colonic fraction may be responsible. Polyphenols, particularly proanthocyanidins from grape peel and catechins from tea, have been shown to inhibit fat-digesting enzymes, including pancreatic lipase [37]. Therefore, the compounds identified in the CF by HPLC-DAD-MS (especially procyanidins) may underlie the observed antiobesity potential.

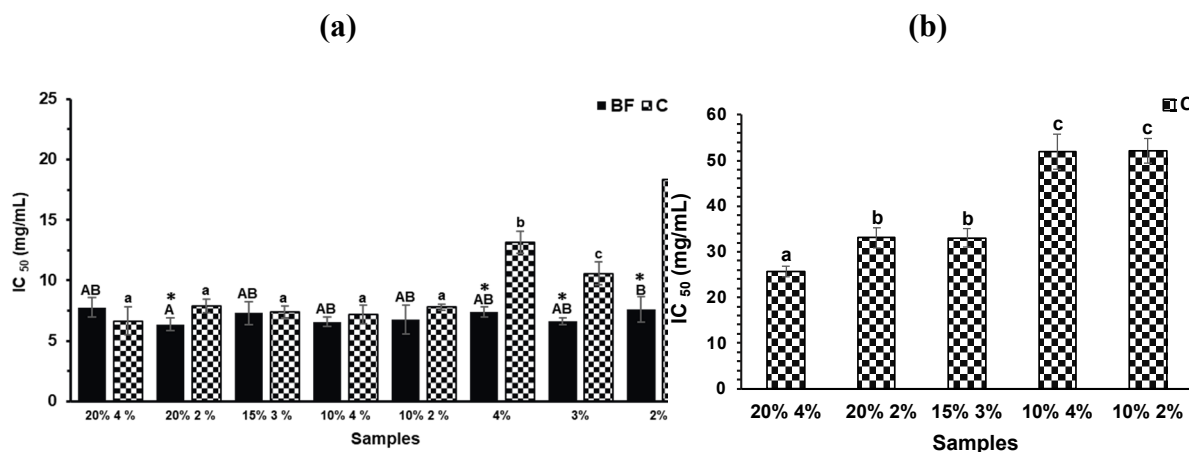


Figure 4. (a) α -glucosidase inhibition of the Bioaccessible and Colonic Fractions (BFs and CFs, respectively) of the different TGP biscuits (TGP% sucralose%: 20% 4%, 20% 2%, 15% 3%, 10% 4% and 10% 2%) and their control biscuits (sucralose%: 4%, 3%, 2%). (b) Pancreatic lipase inhibition of the Colonic Fractions (CFs) of the different formulations of TGP biscuits. Bars denote the mean values and error bars the standard deviation. Different lowercase letters represent significant differences between the CFs of the different biscuit formulations according to Tukey test ($p < 0.05$). Different capital letters represent significant differences between the BF of the different biscuit formulations according to Tukey test ($p < 0.05$). Asterisks (*) indicates significant differences between the CF and BF of the same biscuit formulation (T-test, $p < 0.05$).

2.5. Anti-Inflammatory Capacity

The anti-inflammatory activity of the biscuit samples was evaluated by measuring the inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophages (Figure 5). No significant differences ($p > 0.05$) were observed between the bioaccessible fractions (BFs) of biscuits with and without TGP in terms of NO inhibition. Furthermore, neither BF demonstrated a significant reduction ($p > 0.05$) in NO levels compared to the positive control (LPS). In contrast, the CF of the 20% 4% formulation showed significant differences ($p < 0.05$) by reducing NO production compared to the CF of its control biscuit (4%) and the positive control (LPS). This result suggests that the phenolic compounds provided by the TGP may play a role in modulating NO production [38,39]. Previous studies have shown that phenolic compounds possess anti-inflammatory capacity by reducing NO production under inflammatory conditions [39]. Specifically, in line with the present results, it has been reported that a hydro-alcoholic-acid extract of Tannat grape pomace peel reduces NO production in RAW 264.7 macrophages exposed to LPS stimulation [40], as well as the bioaccessible compounds of this extract [41]. A possible explanation may be that certain compounds, such as polyphenols, could have the capacity to modify the expression or functionality of Nitric Oxide Synthase (NOS), which is the enzyme involved in NO production [42]. Among the various phenolic compounds, the procyanidin trimer, could be responsible for intervening in this metabolic pathway, as it has been reported for its ability to influence the expression of different nitric oxide synthase isoforms [43,44]. In addition, anthocyanins, another prominent group of phenolic compounds in TGP, have been reported to have anti-inflammatory activity. Studies suggest that anthocyanins can modulate inflammatory responses by inhibiting the production of NO through downregulation of NOS expression [45]. Therefore, the presence of anthocyanins in the CF of the biscuits could further explain the observed reduction in NO production, as their synergistic action with other phenolic compounds may contribute to the anti-inflammatory effects seen in this study [46]. Further studies are needed to confirm this hypothesis.

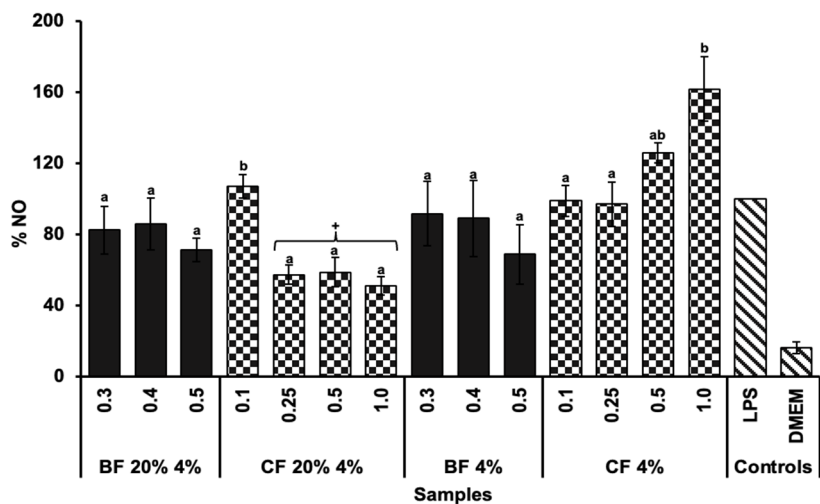


Figure 5. LPS (1 µg/mL)-induced NO production in RAW 264.7 cells treated with the Bioaccessible and Colonic Fractions (BF and CF, respectively) of biscuit with 20% TGP and 4% sucralose (20% 4%) and its control biscuit (4%) without TGP. Bars represent the mean values and error bars represent the standard error. Different letters denote significant differences between concentrations (mg/mL) of the same sample (Tukey, $p < 0.05$). Asterisks (*) indicates significant differences between the same concentrations of BF and CF (T-test, $p < 0.05$). +Indicates significant differences between the sample and the positive control (LPS) (T-test, $p < 0.05$).

3. Materials and Methods

3.1. Materials and Reagents

Analytical grade solvents and reagents were employed for the physicochemical analyses, *in vitro* digestion and cell experiments. The buffer salts, dimethyl sulfoxide (DMSO), Folin reagent, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fluorescein (FL) disodium salt, 2,2'-azobis(2-methylpropionamide) dihydrochloride (AAPH), α -glucosidase, 4-methylumbelliferyl- α -D-glucopyranoside (4-MUG), pancreatic lipase, 4-methylumbelliferyl oleate (4-MUO), human α -amylase (1500 U/mL), porcine pepsin (25,000 U/mL), porcine pancreatin (800 U/mL), bovine bile, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCFH-DA) and lipopolysaccharide from E. coli O55 (LPS) were sourced from Sigma-Aldrich (St. Louis, MO, USA).

Normal human colon cells (CCD 841 CoN) and mouse macrophage cells (RAW 264.7) were procured from the American Type Culture Collection (ATCC, Manassas, VA, USA). CCD 841 CoN cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% (*v/v*) heat-inactivated fetal bovine serum (FBS) and 1% (*v/v*) antibiotics (penicillin-streptomycin, 1:1), all supplied by Gibco Laboratory (Invitrogen Co., Grand Island, NY, USA), whereas RAW 264.7 cells were supplemented with 10% (*v/v*) heat-inactivated fetal bovine serum (FBS).

3.2. Samples

Five biscuit formulations were developed using a two-factorial design with central points, following the procedure described by Olt et al. [5]. The formulations varied in their content of TGP and sucralose, as follows: 20% TGP with 4% sucralose, 20% TGP with 2% sucralose, 15% TGP with 3% sucralose, 10% TGP with 4% sucralose, and 10% TGP with 2% sucralose. All percentages were expressed on a total wet dough basis.

TGP was obtained fresh from Bouza wine cellar (Montevideo, Uruguay) immediately after the winemaking process. The pomace was dried in a conventional oven at 50 °C for 24 hours, ground using a household electric coffee grinder and stored at -20 °C in a freezer until use [40].

Biscuits were formulated as previously reported by Olt et al. [5], using wheat flour, sunflower oil, baking powder, eggs, sucralose, salt and TGP. All ingredients were sourced from local suppliers in Montevideo, Uruguay. The dough from each formulation was rolled out to a uniform thickness of approximately 5 mm and cut into circular discs with a diameter of 4 cm. Biscuits were baked in a conventional oven at 180 °C for 12 minutes, cooled to room temperature, ground using a household grinder, and stored at -20 °C until further analysis.

3.3. Simulated Digestion of the Biscuits

The *in vitro* gastrointestinal digestion simulation of the biscuits was carried out using the INFOGEST protocol [47]. The five biscuits formulations (TGP% sucralose%: 20% 4%, 20% 2%, 15% 3%, 10% 4% and 10% 2%) and their control biscuits (without TGP) (sucralose%: 4%, 3% and 2%), were digested in duplicate. Firstly, biscuits were subjected to the oral phase being diluted with simulated salivary and human α -amylase at pH 7. Samples were incubated with shaking for 2 minutes in a water bath at 37 °C. For the gastric phase, simulated gastric fluid with porcine pepsin and lipase were added to the oral sample at pH 3. Samples were incubated with shaking for 2 h in a water bath at 37 °C. For the small intestinal phase, the gastric samples were added with the simulated intestinal fluid, pancreatin and bile salts at pH 7. The samples were also incubated with shaking for 2 h in a water bath at 37 °C. Finally, enzymatic inactivation was carried out followed by centrifugation (9,000 rpm, 10 min, 4 °C). Two fractions were obtained per biscuit: the soluble fraction, referred to as the Bioaccessible Fraction (BF), consisting of components potentially absorbable; and the insoluble fraction, referred to as the Colonic Fraction (CF), comprising unabsorbed compounds that may reach the colon. The BFs were used directly after the *in vitro* gastrointestinal digestion simulation, whereas the CFs were lyophilized and stored at -20 °C until further analysis. A digestion assay without biscuits (blank) was conducted as a control.

3.4. Identification of Phenolic Compounds in the Colonic Fraction

Phenolic compounds extraction was performed following a modified protocol based on Peña-Vázquez et al. [48]. Briefly, 50 mg of the colonic fraction (CF) from the biscuit containing 20% TGP and 4% sucralose (20% 4%), its corresponding control biscuit (4% sucralose) and the TGP powder were mixed with 1 mL of a methanol:acidified water solution (80:20, *v/v*). Samples were subjected to sonication for 90 minutes, vortexed for 1 minute and then centrifuged at 9,500 rpm for 10 minutes at 4 °C. The supernatant was collected, and the residue was re-extracted using 0.5 mL of the same solvent under identical conditions. The supernatants from both steps were combined and stored at -20 °C for subsequent HPLC-DAD-MS analysis, following the methodology described by Olt et al. [5].

HPLC-DAD-MS analyses were performed on a Kinetex C18-EVO reverse-phase column (Phenomenex) maintained at 35 °C. The mobile phase consisted of 0.1% trifluoroacetic acid in water (A) and acetonitrile (B) with the following gradient elution program: 0–100% A for 3 min; 4–30% B over 50 min; 30–98% B over 5 min; followed by 2 min of isocratic elution at 98% B. The flow rate was set at 1.3 mL/min. Detection was carried out at 280 nm. The HPLC system was coupled to a Shimadzu Triple Quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source (Shimadzu, Tokyo, Japan). This approach was adopted to enable relative comparisons among samples rather than absolute quantification.

3.5. Total Phenolic Content

Total phenolic content (TPC) in the gastrointestinal simulated fractions—BFs and CFs—was determined using the Folin–Ciocalteu method [40]. Absorbance was measured at 750 nm after 30 minutes of incubation in the dark, using a microplate reader (Multiskan FC, Thermo Scientific, Massachusetts, USA). Gallic acid was used as the calibration standard, and results were expressed as milligrams of gallic acid equivalents (mg GAE) per gram of sample.

For TPC analysis, BF_s were used directly after the *in vitro* digestion process. CF_s, on the other hand, were resuspended in a DMSO:distilled water solution (1:1, *v/v*) to enhance the extraction of phenolic compounds. This extraction procedure was selected based on preliminary tests with different DMSO:water proportions (data not shown).

3.6. Antioxidant Capacity

The antioxidant capacity was assessed using the ABTS assay, which evaluates single electron transfer (SET) capacity, and the ORAC-FL assay, which evaluates hydrogen atom transfer (HAT) capacity [40]. For the ABTS assay, absorbance was recorded at 750 nm after 10 minutes of incubation in the dark using a microplate reader (Multiskan FC, Thermo Scientific, Massachusetts, USA). For the ORAC-FL assay, fluorescence ($\lambda_{\text{excitation}} = 485 \text{ nm}$, $\lambda_{\text{emission}} = 520 \text{ nm}$) was recorded every minute for 80 minutes at 37 °C using a Varioskan™ Lux microplate reader (SkanIt RE 5.0 software, Thermo Scientific, Waltham, MA, USA). In both assays, Trolox was used as the calibration standard, and results were expressed as μmol of Trolox equivalents (TE) per gram of sample. BF_s were used as obtained after the *in vitro* digestion, while CF_s were extracted as described in section 3.5.

To evaluate intracellular antioxidant activity, the inhibition of reactive oxygen species (ROS) production under induced oxidative stress conditions was assessed in CCD 841 CoN cells (normal human colon epithelial cells), following the protocol by Fernández-Fernández et al. [29] with minor modifications. BF_s (100 mg/mL) were used directly, while CF_s were prepared in ethanol (95%):water (EtOH:H₂O, 80:20 *v/v*; final EtOH concentration 8% in the well). Sample solutions were filtered and diluted in DMEM-P/S without fetal bovine serum (FBS). Cells were seeded at 1×10^4 cells/well (100 μL /well) in a sterile 96-well translucent microplate with lid and incubated at 37 °C for 24 hours. Afterwards, cells were pre-treated with 100 μL of different concentrations of filtered samples (BF_s and CF_s) at 37 °C for 24 h. Cells were incubated with DCFH-DA probe (5 mg/mL in DMSO) for 30 min after supernatant removal. Once the 30 min incubation ended and cells were washed with PBS, 100 μL of filtered samples with the oxidative agent AAPH (1 mM) were added to each well and incubated at 37 °C for 2 h. Intracellular ROS production was quantified by measuring fluorescence ($\lambda_{\text{excitation}} = 485 \text{ nm}$, $\lambda_{\text{emission}} = 525 \text{ nm}$) using a Varioskan™ Lux microplate reader (SkanIt RE 5.0 software, Thermo Scientific, Waltham, MA, USA). Data normalization was performed using the MTT assay [41]. Positive control presented 1 mM AAPH in DMEM-P/S without FBS which represented 100% ROS formation. Negative control presented DMEM-P/S without FBS. All determinations were performed in triplicate and in 3 different cell passages.

3.7. Antidiabetic and Antiobesity Capacity

The antidiabetic and antiobesity capacities of the samples were evaluated through enzymatic inhibition assays targeting α -glucosidase and pancreatic lipase, respectively. In both assays, fluorescence ($\lambda_{\text{excitation}} = 360 \text{ nm}$, $\lambda_{\text{emission}} = 460 \text{ nm}$) was measured using a Varioskan™ Lux microplate reader (SkanIt RE 5.0 software, Thermo Scientific, Waltham, MA, USA) after 30 minutes of incubation at 37 °C. α -Glucosidase inhibition was assessed using 4-methylumbelliferyl- α -D-glucopyranoside (4-MUG) as the substrate, while pancreatic lipase inhibition was evaluated using 4-methylumbelliferyl oleate (4-MUO). The degree of inhibition was expressed as the concentration of sample required to inhibit 50% of the enzyme activity (IC_{50} , mg/mL) [49]. BF_s were used as obtained after *in vitro* digestion, while CF_s were extracted as described in section 3.5.

3.8. Anti-Inflammatory Capacity

To evaluate the anti-inflammatory capacity of samples, the Nitric Oxide (NO) production in RAW 264.7 cells (macrophages from mouse tumor) was determinate as an indicator of chronic inflammation [40]. RAW 264.7 cells were seeded at 8×10^4 cells/well (100 μL /well) in a sterile 96-well translucent microplate with lid and incubated at 37 °C for 24 h (until confluence). Cells were pre-treated with different concentrations of filtered samples (100 μL /well), prepared as described for

intracellular ROS formation assay (section 3.6.). After 24 h, supernatant was removed and cells were treated for 24 h with 150 μ L of filtered samples with LPS (1 μ g/mL) as an inducer of inflammation. After incubation, 100 μ L of cell supernatants were transferred to another 96-well plate and mixed with 100 μ L of Griess reagent which reacts with organic nitrites. After 15 min incubation in the dark at room temperature, absorbance at 550 nm was measured in a VarioskanTM Lux (SkanIt RE 5.0 software, Thermo Scientific, Waltham, MA, USA). To normalize data by the viable cells' number, MTT assay was performed by adding 20 μ L/well of MTT (6mM) reagent and incubating for 30 min [41]. Positive control presented 1 μ g/mL LPS in DMEM-P/S without FBS which represented 100% of NO production. Negative control presented DMEM-P/S without FBS. All determinations were performed in triplicate and in three different cell passages.

3.9. Statistical Analysis

Three independent experiments were performed for each assay. Results were expressed as mean \pm standard deviation. Statistical differences between the mean values of the studied parameters of the different samples were determined by the Tukey test ($p < 0.05$). In cell studies, the statistical differences between mean values of samples were determined by the T-test ($p < 0.05$). The program used for statistical analyses was Infostat v. 2020.

4. Conclusions

The results from this research provide valuable insights into the impact of *in vitro* gastrointestinal digestion on the release and retention of phenolic compounds from biscuits formulated with varying percentages of Tannat grape pomace (TGP). The bioaccessible fractions (BFs) showed similar levels of bioactivity among themselves and compared to their respective control biscuits ($p > 0.05$). In contrast, the colonic fractions (CFs) of biscuits with higher TGP content (20% and 15%) exhibited significantly greater antioxidant and enzyme inhibitory activities ($p < 0.05$). These findings indicate that phenolic compounds may be selectively retained in the CF during digestion.

In particular, the CF of the biscuit containing 20% TGP and 4% sucralose showed significantly higher inhibition of lipase and α -glucosidase compared to the rest of the biscuits ($p < 0.05$). Furthermore, the CF of the biscuit with 20% TGP and 4% sucralose showed a significant reduction ($p < 0.05$) of intracellular Reactive Oxygen Species (ROS) and Nitric Oxide (NO) formation compared to the CF of the control biscuit, as well as compared to the BFs, suggesting the potential of phenolic compounds to counteract oxidative and nitrosative stress. HPLC-DAD-MS analysis revealed the presence of procyanidin trimers in the CF, which may contribute to its observed bioactivities.

This study expands current knowledge by providing the first evidence of such behavior in biscuits enriched with Tannat grape pomace, combining *in vitro* gastrointestinal digestion, cell assays and phenolic compound identification. It underscores the critical role of matrix–compound interactions in modulating phenolic bioaccessibility and highlights the potential of TGP to contribute bioactive compounds to the colonic environment. Further studies are needed to identify the phenolic compounds in the BFs, as well as to investigate the metabolism of CFs in the colon and the phenolic compound transformation by intestinal microbiota.

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Abbreviations

The following abbreviations are used in this manuscript:

4-MUG	4-methylumbelliferyl- α -D-glucopyranoside
4-MUO	4-methylumbelliferyl oleate
AAPH	2,2'-Azo- bis (2-methylpropionamidine) dihydrochloride
ABTS	2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
CCD 841 CoN	Cell line isolated from colon tissue of a healthy donor
FBS	Fetal bovine serum
GAE	Gallic acid equivalents
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimetilsulfoxide
HAT	Hydrogen atom transfer
HPLC-DAD-MS	High-performance liquid chromatography with diode array detection and mass spectrometry
IC ₅₀	Inhibitory concentration 50%
LPS	Lipopolysaccharide
MTT	3-(4,5-dimethylthiazole-y)-2,5-diphenyltetrazolium bromide
NO	Nitric oxide
ORAC	Oxygen radical absorbance capacity
RAW 264.7	Mouse macrophage cell line
ROS	Reactive oxygen species
TPC	Total phenolic content
TGP	Tannat grape pomace
TROLOX	6-hydroxy-2,5,7,8-tetramethylch-roman- 2-acid
SET	Single electron transfer

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