



## Digestibility of sustainable muffins enriched with Tannat grape pomace

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### ABSTRACT

**Background:** *Vitis vinifera* cv. Tannat, Uruguayan emblematic grape variety, is rich in bioactive phenolics, many of which remain in its abundant byproduct, grape pomace. This makes Tannat grape pomace (TGP) a promising functional ingredient.

**Objective:** To analyze the residual phenolic compounds and bioactive properties of TGP after digestion and incorporate it into a muffin formulation as a functional ingredient.

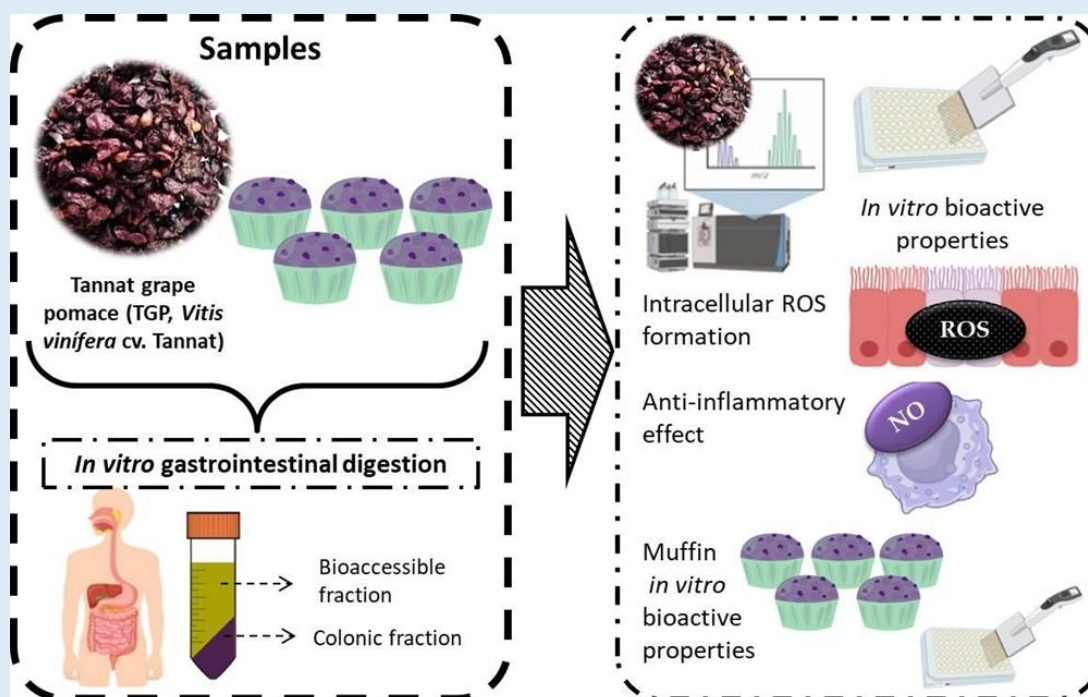
**Methods:** *In vitro* digestion simulation of TGP and muffin was assessed following the INFOGEST protocol. UHPLC-MS/MS performed phenolic compound identification. Total phenolic compounds (Folin-Ciocalteu method), antioxidant capacity (ABTS and ORAC-FL methods), and antidiabetic capacity ( $\alpha$ -glucosidase activity inhibition) were assessed, as well as antioxidant and anti-inflammatory effects on cells. TGP was added to a muffin formulation.

**Results:** After *in vitro* digestion, bioaccessible (BF) and colonic fractions (CF) were obtained. UHPLC-MS/MS results of TGP BF showed the presence of phenolic acids, flavan-3-ols, flavonols, and anthocyanins. TGP BF showed TPC, ABTS, ORAC-FL, and  $\alpha$ -glucosidase lower values than TGP CF, indicating most of the TGP phenolic compounds may be retained by the grape matrix. TGP BF and CF showed intracellular antioxidant effect on CCD-841CoN colon cells and anti-inflammatory effect on RAW264.7 macrophages. The addition of TGP to the muffin formulation demonstrated increased bioactive properties ( $p < 0.05$ ). However, the BF of the TGP muffin showed similar bioactive properties ( $p > 0.05$ ) with the control muffin (without TGP), while the CF of the TGP muffin presented higher than the control muffin ( $p < 0.05$ ).

**Novelty of the Study:** This is the first study on TGP phenolic compound identification and bioactive properties after *in vitro* simulation of digestion, as well as its incorporation in muffins and confirmation of its bioactive properties after digestion. The most novel findings of the present study underline the colonic fraction of TGP and muffin with potential antioxidant and anti-inflammatory effects that may promote colon health. These findings contribute to the development of foods with confirmed functionality.

**Conclusion:** The persistence of phenolic compounds and bioactive properties from TGP after digestion was confirmed, showing potential as a functional ingredient. However, these compounds appear to be non-bioaccessible when incorporated into a muffin formulation. Future studies on the microbiota effect of the TGP muffin CF are needed to state the potential as a functional food.

**Keywords:** digestibility, functional foods, phenolic compounds, sustainable muffins, Tannat grape pomace



Graphical Abstract: Digestibility of sustainable muffins enriched with tannat grape pomace.

## INTRODUCTION

The contribution of a functional ingredient to human health depends not only on its bioactive compounds but also on how they are released, absorbed, and metabolized once incorporated into a food and consumed [1]. In the case of phenolic compounds, even high concentrations in foods may not necessarily result in physiological benefits [2]. Their actual contribution depends on several factors, including the food matrix into which they are incorporated and the fraction that is released and absorbed in the small intestine, which generally represents only a minor proportion [2]. In addition, another fraction reaches the colon, where the gut microbiota metabolizes it into metabolites with relevant biological activity [3].

Grape pomace (GP), the primary solid byproduct generated during winemaking, is composed primarily of skins and seeds and has been identified as a potential functional ingredient due to its high content of dietary fiber and phenolic compounds [4]. Its fiber profile is dominated by insoluble fiber, mainly cellulose and hemicellulose, whereas soluble fiber includes pectins,  $\beta$ -glucans, gums, mucilages, oligosaccharides, and inulin [5,6]. Among various red grape varieties, Tannat (*Vitis vinifera* cv. Tannat), widely cultivated in Uruguay, is distinguished by its high concentration and complexity of phenolic compounds [7]. Its pomace (TGP) includes malvidin-3-O-(6-p-coumaroyl) glucoside, procyanidin trimer, malvidin-3-O-glucoside, (-)-epicatechin, and p-coumaroyl hexose as the primary phenolic compounds, as well as a high dietary fiber content of about 64% [8].

The incorporation of GP into bakery products, such as muffins, has been explored as a strategy to improve nutritional, technological, and sensory quality, with positive effects on fiber, polyphenol content, and antioxidant capacity [9-11]. Although some studies have evaluated the bioaccessibility of phenolic compounds from Tannat grape byproducts in other food matrices, such as biscuits [12] and yogurt [13], information

specifically addressing muffins fortified with this byproduct remains limited. In this context, the present study aimed to valorize TGP as a potential functional ingredient by analyzing the residual phenolic compounds and bioactive properties after digestion, as well as to evaluate the bioactive properties of a muffin containing TGP.

## MATERIALS AND METHODS

**Materials:** Potassium persulphate was purchased from J. T. Baker, while Sigma-Aldrich (St. Louis, MO, USA) provided standards [6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, and acarbose]; reagents for *in vitro* bioactivity assays [fluorescein disodium salt; 2,20-azobis (2-methylpropionamidine) dihydrochloride (AAPH); 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS); Folin reagent;  $\alpha$ -glucosidase from rat intestine acetone powder; 4-methylumbelliferyl- $\alpha$ -D-glucopyranoside]; and for *in vitro* simulation of digestion (human  $\alpha$ -amylase, pancreatic lipase, porcine pepsin, porcine pancreatin, bovine bile).

American Type Culture Collection (ATCC, Manassas, VA, USA) provided normal human colon cells (CCD 841 CoN) and mouse macrophages (RAW 264.7). CCD 841 CoN and RAW264.7 cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with heat-inactivated fetal bovine serum (FBS, 20 and 10 % v/v, respectively) and 1% (v/v) antibiotics (penicillin-streptomycin, 1:1) supplied by Gibco Laboratory (Invitrogen Co., Grand Island, NY, USA).

### Tannat grape pomace preparation and muffin

**elaboration:** Bouza winecellar (Montevideo, Uruguay) provided Tannat grape pomace (TGP). A conventional oven (50 °C) was used to dry the TGP, achieving a constant weight at 24 h, and milled in a domestic coffee mill [8] using the obtained powder for the muffin elaboration. To elaborate, the muffins TGP, wheat flour,

baking powder, sweetener (stevia), eggs, sunflower oil, cow milk, and vanilla were employed and were purchased from local stores in Montevideo (Uruguay).

**Phenolic compounds and antioxidant capacity (ABTS and ORAC-FL):** The identification of the phenolic compounds in TGP and the bioaccessible fraction was performed by UHPLC-MS/MS [13]. Briefly, freeze-dried samples were dissolved in H<sub>2</sub>O:MeOH (50:50, v/v; 10 mg/mL) and analyzed using a Thermo Ultimate™ 3000 HPLC system (Thermo Scientific, Sunnyvale, CA, USA) coupled to a Q-Exactive™ hybrid quadrupole–orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a heated electrospray ionization (HESI-II) source and a Thermo Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS) software. UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm; Waters, Milford, MA, USA) was used for separation with a water–acetonitrile gradient mobile phase at a flow rate of 0.3 mL/min. Phenolic compounds were identified by accurate mass measurements of precursor and product ions, isotope pattern analysis, and comparison with reference standards when available.

Total Phenolic Content (TPC) and overall antioxidant capacity were assessed following the procedures of Fernández-Fernández et al. [14]. Briefly, samples were prepared in DMSO in distilled water as described by Fernández-Fernández et al. [14]. For ABTS and ORAC-FL assays, Trolox calibration curves (0.25–1.5 mM for ABTS; 0.1–0.8 mM for ORAC-FL) were constructed, and results were expressed as µmol Trolox equivalents (TE)/g muffin. For ABTS and ORAC-FL assays, Trolox calibration curves (0.25–1.5 mM for ABTS; 0.1–0.8 mM for ORAC-FL) were constructed, and results expressed as µmol Trolox equivalents (TE)/g muffin. All assays were conducted at least in triplicate. For the Folin-Ciocalteu and ABTS methods, absorbance was measured at 750 nm 30 and 10 minutes later, respectively, using a Thermo Scientific Multiskan FC microplate reader (Waltham, MA, USA). For the ORAC-FL assay,

fluorescence was monitored ( $\lambda_{\text{excitation}} = 485 \text{ nm}$ ,  $\lambda_{\text{emission}} = 520 \text{ nm}$ ) for 80 minutes with a Varioskan™ Lux microplate reader (SkanIt RE 5.0 software, Thermo Scientific, Waltham, MA, USA).

**α-glucosidase inhibition:** The inhibition of α-glucosidase enzymatic activity was evaluated as described by Fernández-Fernández et al. [14], as it is involved in carbohydrate digestion. Briefly, α-glucosidase was extracted from rat intestinal acetone powder in NaCl 0.9%, centrifuged for 30 min at 10.000 g, and the supernatant was diluted (1/10). The enzymatic activity was measured using a substrate (4-methylumbelliferyl-α-D-glucopyranoside, 4-MUG) at 37 °C for 30 minutes by fluorescence ( $\lambda_{\text{ex}} = 360 \text{ nm}$  and  $\lambda_{\text{em}} = 460 \text{ nm}$ ) in a fluorimeter microplate reader (Varioskan™ Lux, SkanIt RE 5.0 software, Thermo Scientific, Waltham, MA, USA).

**In vitro simulation of gastrointestinal digestion of TGP and muffins:** The *in vitro* digestion simulation of TGP, TGP muffin, and control muffin (without TGP) was carried out by the INFOGEST protocol [16]. Three independent digestions were performed for each sample. Briefly, samples were rinsed with simulated salivary fluid and human α-amylase (1500 U/mL, pH 7) and incubated in a water bath (2 min) with shaking at 37 °C for the oral phase. Then, simulated gastric fluid, porcine pepsin (25,000 U/mL), and pancreatic lipase (pH 3) were added to the oral mix, and it was incubated for 2 hours in a water bath shaking at 37 °C to stimulate the gastric phase. Afterwards, simulated intestinal fluid, porcine pancreatin (800 U/mL), and bile salts (pH 7) were added to the gastric samples, and incubated for two hours in a water bath at 37 °C with shaking for the small intestinal phase. Finally, enzymatic inactivation was carried out in a water bath at 90 °C for 10 min, followed by 9000 rpm centrifugation for 10 min at 4 °C, obtaining two fractions: the bioaccessible fraction (BF, supernatant) and the colonic fraction (CF, precipitate). Following the *in vitro* gastrointestinal digestion simulation, the BFs were used

directly for all the analyses. However, for UHPLC-MS/MS, the samples were freeze-dried; the CFs were lyophilized and preserved at  $-20^{\circ}\text{C}$  until analysis. Digestion control was conducted without any samples.

**TGP cell studies:** Antioxidant and anti-inflammatory effects were assessed on normal human colon cells (CCD-841CoN) and mouse macrophages (RAW264.7), respectively. TGP BF was used as obtained from an *in vitro* simulation of digestion and diluted. TGP CF was prepared in EtOH: H<sub>2</sub>O (80:20 v/v). Both samples were tested on the cells by diluting in DMEM+P/S without FBS. Cell studies were performed in triplicate and in three different cell passages.

The intracellular ROS formation was measured under oxidation (AAPH, 1 mM)-induced conditions, as described by Fernández-Fernández et al. [13], with the modifications reported by Olt et al. [12]. CCD-841CoN cells were seeded ( $1 \times 10^4$  cells/well, 100  $\mu\text{L}$ /well) in 96-well plates with a lid, incubated at  $37^{\circ}\text{C}$  for 24 h, treated with the samples (100  $\mu\text{L}$ ) at  $37^{\circ}\text{C}$  for 24 h followed by supernatant removal. Then, cells were incubated with the probe DCFH-DA for 30 min, washed twice with PBS, incubated with 100  $\mu\text{L}$  of sample and AAPH (1 mM)  $37^{\circ}\text{C}$  for 2 h, measured using a Varioskan™ Lux microplate reader (SkanIt RE 5.0 software, Thermo Scientific, Waltham, MA, USA) ( $\lambda$  excitation = 485 nm,  $\lambda$  emission = 525 nm), and normalized with MTT absorbance results, followed by taking the positive control (AAPH) as 100 % ROS formation.

Nitric oxide (NO) production was measured under inflammatory (lipopolysaccharide, LPS, one  $\mu\text{g}/\text{mL}$ )-induced conditions, as described by Fernández-Fernández et al. [13]. RAW264.7 cells were seeded ( $8 \times 10^4$  cells/well, 100  $\mu\text{L}$ /well) in 96-well plates with a lid, incubated at  $37^{\circ}\text{C}$  for 24 h, treated with the samples (100  $\mu\text{L}$ ) at  $37^{\circ}\text{C}$  for 24 h, the supernatant was removed, incubated with 150  $\mu\text{L}$  of sample and LPS (1  $\mu\text{g}/\text{mL}$ )  $37^{\circ}\text{C}$  for 24 h, 100  $\mu\text{L}$  cell supernatant transfer to another translucent microplate of 96 wells, added with 100  $\mu\text{L}$  of

Griess reagent, measured at 550 nm after 15 min incubation at room temperature in the dark by the same equipment (Varioskan™ Lux microplate reader), and normalized with MTT absorbance results, followed by taking the positive control (LPS) as 100 % NO production.

**Experimental design of muffin formulations:** Five formulations were proposed based on a factorial experimental design with central points [17]. The two factors were the percentage of TGP (7, 11 and 15%) and the stevia content (0.65, 0.75, and 0.85 g). The selected TGP levels were intended to enable the muffins to meet the nutritional claim of “source of fiber” based on its reported fiber content of 64% [8]. Control muffins without TGP with the respective stevia contents were also elaborated for comparison purposes.

**Statistical analysis:** All assays were conducted in triplicate, and results were expressed as the mean  $\pm$  standard deviation. Data were analyzed by analysis of variance (ANOVA), with mean differences evaluated using the Tukey test ( $p < 0.05$ ) for more than two samples. Pairwise comparisons between two samples were assessed using Student's t-test ( $p < 0.05$ ). Statistical analyses were performed with the Infostat v. 2015 program (Universidad Nacional de Córdoba, Córdoba, Argentina).

## RESULTS AND DISCUSSION

**Digestibility of TGP through identification of phenolic compounds and *in vitro* bioactivity:** UHPLC-MS/MS results of TGP and its bioaccessible fraction (Table 1) showed the presence of phenolic acids (p-coumaroyl hexose, gallic, coumaric, and trans-caftaric acids) and flavan-3-ols (catechin, epicatechin, epigallocatechin, procyanidin dimers and trimers, and their respective galloylated derivatives, as well as prodelphinidin dimers). Also, flavonols (quercetin and its derivatives, rutin, rhamnetin, isorhamnetin, myricetin, laricitrin and syringetin) and anthocyanins (cyanidin, delphinidin,

malvidin, peonidin, petunidin, and its respective derivatives) were identified. The main phenolic compounds present in TGP were malvidin, malvidin-3-O-(6'-p-coumaroyl) glucoside, epicatechin, catechin, and malvidin-3-(6'-O-acetylglucoside) in descending order of content based on relative areas. Gallic acid, catechin and epicatechin, quercetin, malvidin and malvidin-3-O-(6'-p-coumaroyl) glucoside, were the main phenolic acid, flavan-3-ol, flavonol and anthocyanins, respectively, present in TGP. The results agree with previous studies on TGP where trans-caftaric acid, procyanidin dimers and trimers, catechin and epicatechin, quercetin derivatives, and malvidin derivatives were found [8], the reported by Fariña et al. [18] on TGP extracts and Rajchman et al. [19] on TGP PLE extracts. Moreover, most of the anthocyanins identified in TGP were also found in Tannat grape skin (TGS) from pomace [13] and its extract [20], as well as in Tannat wines [21]. Also, in agreement with the present results, oligo-polymers are the main flavan-3-ols in Tannat grape seeds followed by dimers [22].

After *in vitro* digestion, most of the phenolic

compounds present in TGP remained bioaccessible, except for prodelphinidin dimer 3, procyanidin hexamer galloylated, and delphinidin-3-O-(6'-p-coumaroyl) glucoside that were not detected. The latter could indicate that they may have been degraded or changed into another phenolic compound [23] or may be present in the CF as delphinidin-3-O-(6'-p-coumaroyl) glucoside was also identified in the colonic fraction of TGP [12]. Moreover, the main compounds present in the BF were the same as in TGP but in different order of content based on relative areas: malvidin, epicatechin, catechin, malvidin-3-(6'-O-acetylglucoside), and malvidin-3-O-(6'-p-coumaroyl) glucoside. The observed results may be attributed to the degradation of malvidin-3-O-(6'-p-coumaroyl) glucoside and the relative stability of epicatechin and catechin during digestion [23-24]. Consistent with Nieto et al. [23], a reduction in procyanidins was also detected after digestion. To our knowledge, this is the first work to identify bioaccessible compounds from TGP, highlighting those with potential intestinal absorption.

**Table 1.** UHPLC-MS/MS phenolic profile of TGP and its bioaccessible fraction.

| Compound                  | TGP     | TGP BF  | [M + H] <sup>+</sup> | λ <sub>máx</sub> |
|---------------------------|---------|---------|----------------------|------------------|
| <b>Phenolic Acids</b>     |         |         |                      |                  |
| p-Coumaric acid           | 131807  | 537257  | 165                  | 234, 310         |
| Gallic acid               | 406389  | 579401  | 169                  | 273              |
| trans-caftaric acid       | 133914  | 174565  | 313                  | 250, 329         |
| p-coumaroyl hexose 1      | 60556   | 66779   | 325                  | 236, 314         |
| p-coumaroyl hexose 2      | 66605   | 71556   | 325                  | 236, 314         |
| <b>Flavonols</b>          |         |         |                      |                  |
| Isorhamnetin              | 35313   | 58416   | 317                  | 253, 370         |
| Laricitrin                | 25220   | 19014   | 333                  | 254, 374         |
| Malvidin                  | 7098373 | 4748108 | 331                  | 274, 538         |
| Myricetin                 | 24567   | 9011    | 319                  | 253, 372         |
| Quercetin                 | 140402  | 280851  | 303                  | 255, 370         |
| Quercetin-3-O-galactoside | 12944   | 1958    | 465                  | 252, 354         |
| Quercetin-3-O-glucoside   | 127801  | 125356  | 465                  | 256, 354         |
| Rhamnetin                 | 35313   | 58416   | 317                  | 257, 266, 370    |
| Rutin                     | 3408    | 6286    | 611                  | 257, 329         |
| Syringetin                | 38277   | 218278  | 347                  | 216, 308         |
| <b>Other Pigments</b>     |         |         |                      |                  |
| Cyanidin                  | 53746   | 27226   | 288                  | 279, 518         |

| Compound                                  | TGP     | TGP BF  | [M + H] <sup>+</sup> | λ <sub>máx</sub> |
|-------------------------------------------|---------|---------|----------------------|------------------|
| Cyanidin 3-(6-O-acetylglucoside)          | 21459   | 14744   | 491                  | 250, 523         |
| Cyanidin-3-O-(6-p-coumaroyl) glucoside    | 61504   | 8530    | 595                  | 284, 314, 524    |
| Delphinidin-3-(6-O-acetylglucoside)       | 24654   | 6290    | 507                  | 276, 346, 527    |
| Delphinidin-3-O-(6-p-coumaroyl) glucoside | 42048   | n.a.    | 611                  | 282, 313, 531    |
| Malvidin-3-(6-O-acetylglucoside)          | 1718369 | 899538  | 535                  | 278, 350, 530    |
| Malvidin-3-O-(6-p-coumaroyl) glucoside    | 2580164 | 738734  | 639                  | 280, 301, 535    |
| Peonidin                                  | 704455  | 334116  | 302                  | 526              |
| Peonidin-3-(6-O-acetylglucoside)          | 146172  | 51163   | 505                  | 280, 522         |
| Peonidin-3-O-(6-p-coumaroyl) glucoside    | 230696  | 41364   | 609                  | 283, 313, 526    |
| Petunidin                                 | 708726  | 143049  | 354                  | 274, 538         |
| Petunidin-3-(6-O-acetylglucoside)         | 213033  | 40157   | 521                  | 270, 529         |
| Petunidin-3-O-(6-p-coumaroyl) glucoside   | 253194  | 63975   | 625                  | 282, 313, 532    |
| <b>Flavan-3-ols</b>                       |         |         |                      |                  |
| (+)-Catechin                              | 2514813 | 1544220 | 291                  | 233, 278         |
| (-)-epicatechin                           | 2562334 | 1561751 | 291                  | 234, 278         |
| (-)-epigallocatechin                      | 9406    | 5343    | 307                  | 235, 269         |
| (+)-gallocatechin                         | 20989   | 16557   | 307                  | 234, 275         |
| Procyanidin dimer B1                      | 127795  | 107785  | 579                  | 278              |
| Procyanidin dimer B2                      | 409613  | 247961  | 579                  | 278              |
| Procyanidin dimer B3                      | 112270  | 42090   | 579                  | 278              |
| Procyanidin hexamer galloylated           | 2931    | n.a.    | 1883                 | 278              |
| Procyanidin pentamer galloylated          | 18029   | 695     | 1595                 | 278              |
| Procyanidin tetramer digalloylated        | 12083   | 838     | 1459                 | 278              |
| Procyanidin trimer C2                     | 523395  | 240114  | 867                  | 278              |
| Procyanidin trimer digalloylated          | 21875   | 1532    | 1171                 | 278              |
| Prodelphinidin dimer 1                    | n.a.    | 630     | 579                  | 278              |
| Prodelphinidin dimer 2                    | 6952    | 800     | 579                  | 278              |
| Prodelphinidin dimer 3                    | 4385    | n.a.    | 579                  | 278              |

Results are expressed in relative areas. TGP: Tannat Grape Pomace; TGP BF: Tannat Grape Pomace Bioaccessible Fraction.

Regarding the results for TPC and the bioactive properties of TGP (Table 2), the CF exhibited the highest values, indicating that most phenolic compounds from TGP may have been retained during digestion thus remaining in the CF, or the ones released during digestion, were likely retained during digestion and remained in this fraction. Alternatively, compounds released during digestion may have mainly been insoluble or precipitated, thereby accumulating in the CF. Nevertheless, a portion of the phenolics remained bioaccessible, as supported by both the *in vitro* bioactive properties (Table 2) and the UHPLC-MS/MS results (Table

1). These compounds therefore have the potential to be absorbed and to exert health promoting properties at the intestine. Moreover, the TPC and ORAC-FL values were comparable to those reported for the bioaccessible fraction of Tannat grape skin (7.41 mg GAE/g sample and 128.3 μmol TE/g sample, respectively) [13]. In contrast, the bioaccessible fractions of high and low polymeric extracts (HPE and LPE, respectively) from Merlot grape stems obtained by pressurized liquid extraction showed higher TPC and DPPH values [23] compared to the ABTS values observed in the present work. Furthermore, HPE exhibited a smaller decrease in antioxidant capacity

(69%) than the 78% reduction observed for the TGP BF which may be attributed to the low stability of TGP anthocyanins at intestinal pH [25].

As to  $\alpha$ -glucosidase inhibition, the bioaccessible

fraction of TGP showed a lower inhibition capacity (higher  $IC_{50}$  value) than that of Tannat grape skin [13], and also lower than extracts from grape pomace, seeds and skins [26].

**Table 2.** Bioactive properties of TGP fractions obtained after *in vitro* simulation of digestion.

|                                            | Bioaccessible Fraction         | Colonic Fraction                |
|--------------------------------------------|--------------------------------|---------------------------------|
| TPC (mg GAE/g)                             | 7.09 $\pm$ 0.52 <sup>a</sup>   | 41.75 $\pm$ 1.01 <sup>b</sup>   |
| ABTS ( $\mu$ mol TE/g)                     | 100.46 $\pm$ 7.23 <sup>a</sup> | 751.19 $\pm$ 9.09 <sup>b</sup>  |
| ORAC-FL ( $\mu$ mol TE/g)                  | 187.00 $\pm$ 4.31 <sup>a</sup> | 322.87 $\pm$ 14.39 <sup>b</sup> |
| $\alpha$ -glucosidase ( $IC_{50}$ , mg/mL) | 13.61 $\pm$ 2.30 <sup>b</sup>  | 5.21 $\pm$ 0.51 <sup>a</sup>    |

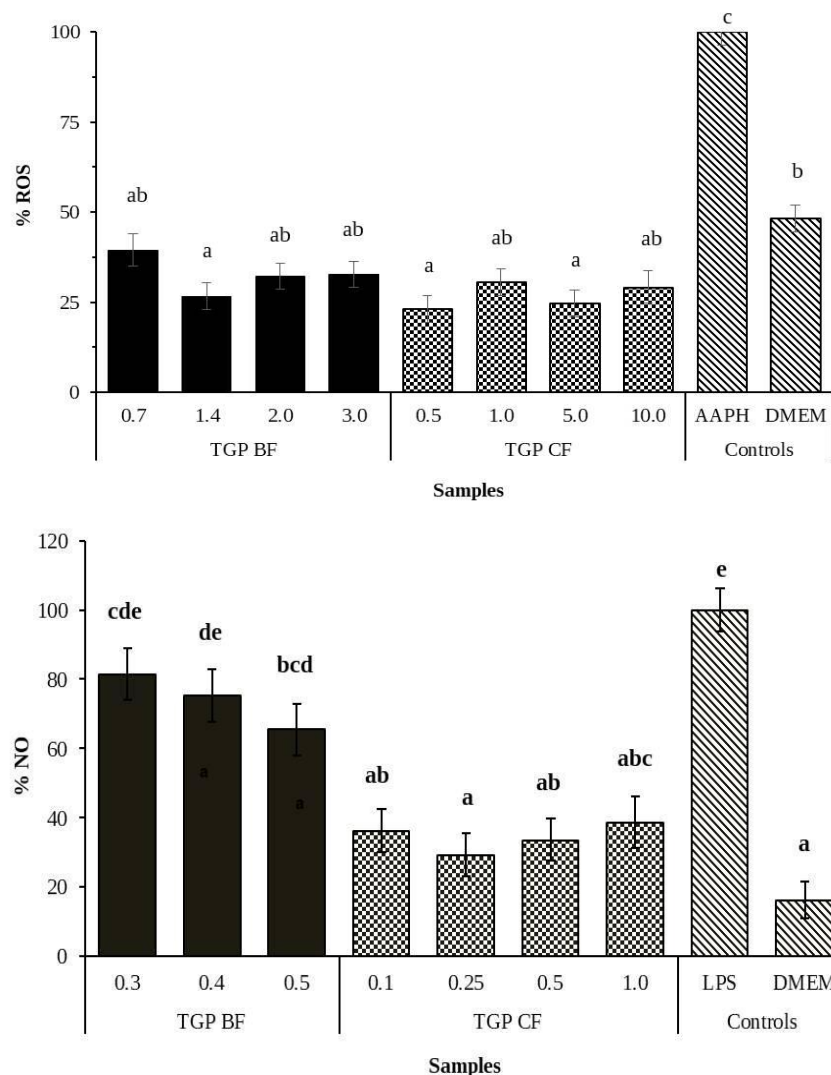
Results are expressed as mean  $\pm$  standard deviation. Different letters in the same row indicate significant differences by t-test ( $p < 0.05$ ).

When assessing the antioxidant effect on intestinal cells, both the BF and CF of TGP markedly inhibited intracellular ROS formation in CCD-841CoN normal human colon cells exposed to oxidative stress by AAPH (1 mM), compared with the positive control (cells treated with AAPH alone) (Figure 1). The phenolic compounds present in both fractions (BF and CF) are likely responsible for the exerted antioxidant effect, as supported by the UHPLC-MS/MS phenolic profile and antioxidant capacity results. These compounds may act by neutralizing peroxy radicals in the extracellular compartment [27], thereby preventing intracellular oxidation of the probe (DCFH-DA). Significant inhibition ( $p < 0.05$ ) was observed only when the samples (BF and CF) were co-administered with AAP. In contrast, no protection was detected when cells were pre-treated with the samples and subsequently exposed to AAPH in their absence. These findings are consistent with previous studies by Olt et al. [12] who reported similar effects in CCD-841CoN treated with TGP biscuits, and by Fernández-Fernández et al. [20], who observed comparable antioxidant behavior in CCD-18Co treated with the BF of a hydro-alcoholic-acid extract of TGP peel. In both studies, inhibition of intracellular ROS formation

occurred only during co-administration with the oxidant, confirming that the antioxidant effect requires the simultaneous presence of the bioactive compounds and the oxidative challenge.

Regarding the anti-inflammatory activity, the CF of TGP markedly reduced NO production in RAW264.7 macrophages compared with the positive control (LPS, 1  $\mu$ g/mL). A similar, though less pronounced, effect was also observed for the BF at the highest concentration tested, maintaining cell viability above 80 %. The results are in line with those reported by Fariña et al. [18], who demonstrated that Tannat pomace extracts modulate NF- $\kappa$ B activity and inhibit IL-8 production in reporter intestinal cells (HT-29- NF- $\kappa$ B-hrGFP) under TNF- $\alpha$ -induced inflammation. They also agree with the inhibition of NO production observed for the bioaccessible fraction of Tannat grape skin [20] and for both bioaccessible and colonic fractions of TGP biscuits [12]. Procyanidins and anthocyanins are likely the main contributors to this anti-inflammatory effect [28-30].

This is the first report on the antioxidant effects of TGP bioaccessible and colonic fractions in intestinal cells and their anti-inflammatory activity in macrophages, supporting TGP's potential as a functional ingredient.



**Figure 1.** Cell assays of TGP after *in vitro* simulation of digestion. A) CCD-841CoN (normal human colon cells) intracellular ROS formation under AAPH (1 mM)-induced oxidation. The assay was performed by treating the cells with TGP bioaccessible and colonic fractions (BF and CF, respectively) for 24 h followed by supernatant removal, incubation with DCFH-DA probe for 30 min, incubation with samples and AAPH (1 mM) for 2 h. DMEM and AAPH (1 mM) were used as negative and positive controls, respectively. B) RAW264.7 (mouse macrophages) nitric oxide production under LPS (1 µg/mL)-induced inflammation. Cells were pre-treated with TGP bioaccessible and colonic fractions (BF and CF, respectively) for 24 h followed by supernatant removal, and incubation with samples and LPS (1 µg/mL) for 24 h. DMEM and LPS (1 µg/mL) were used as negative and positive controls, respectively. The mean values are presented as bars, and the standard errors are presented as the error bars. Different letters state significant differences between concentrations (mg/mL) of all the samples (Tukey,  $p < 0.05$ ).

**TGP application as a potential functional ingredient in muffins:** TGP increased muffin *in vitro* bioactive properties compared to control muffins (duplicate stevia content without TGP,  $p < 0.05$ ). Moreover, muffins with the highest TGP content (15%) exhibited the highest bioactive properties ( $p < 0.05$ , Table 3), which may be

attributed to the phenolic composition of TGP (Table 1). The latter agrees with a report on the nutritional quality improvement of muffins, cereal bars, and biscuits achieved by the addition of Malbec grape pomace [32] and biscuits supplemented with TGP [8].

**Table 3.** *In vitro* bioactive properties of the control and TGP muffins.

| Muffins         |          | TPC<br>(mg GAE/g muffin) |   |                    | ABTS<br>( $\mu\text{mol TE/g muffin}$ ) |   |                   | ORAC-FL<br>( $\mu\text{mol TE/g muffin}$ ) |   |                   | $\alpha$ -glucosidase<br>(% Inhibition) |
|-----------------|----------|--------------------------|---|--------------------|-----------------------------------------|---|-------------------|--------------------------------------------|---|-------------------|-----------------------------------------|
| Control Muffins | 0.65     | 2.48                     | ± | 0.13 <sup>a</sup>  | 3.95                                    | ± | 0.32 <sup>a</sup> | 6.31                                       | ± | 0.75 <sup>a</sup> | 47.0±3.4 <sup>a</sup>                   |
|                 | 0.75     | 2.55                     | ± | 0.10 <sup>a</sup>  | 3.96                                    | ± | 0.30 <sup>a</sup> | 6.21                                       | ± | 0.11 <sup>a</sup> | 45.3±2.4 <sup>a</sup>                   |
|                 | 0.85     | 2.44                     | ± | 0.18 <sup>a</sup>  | 3.20                                    | ± | 0.22 <sup>a</sup> | 10.42                                      | ± | 0.96 <sup>a</sup> | 41.5±2.8 <sup>a</sup>                   |
| TGP Muffins     | 7% 0.65  | 2.72                     | ± | 0.07 <sup>bc</sup> | 7.00                                    | ± | 0.43 <sup>b</sup> | 18.65                                      | ± | 0.53 <sup>b</sup> | 49.5±2.2 <sup>b</sup>                   |
|                 | 7% 0.85  | 2.77                     | ± | 0.31 <sup>b</sup>  | 6.62                                    | ± | 0.89 <sup>b</sup> | 16.52                                      | ± | 1.59 <sup>b</sup> | 57.5±0.2 <sup>bc</sup>                  |
|                 | 11% 0.75 | 2.76                     | ± | 0.12 <sup>c</sup>  | 11.67                                   | ± | 1.04 <sup>c</sup> | 16.87                                      | ± | 1.55 <sup>b</sup> | 53.9±2.6 <sup>bc</sup>                  |
|                 | 15% 0.65 | 3.17                     | ± | 0.55 <sup>d</sup>  | 15.59                                   | ± | 1.08 <sup>d</sup> | 23.92                                      | ± | 2.40 <sup>c</sup> | 62.2±2.4 <sup>d</sup>                   |
|                 | 15% 0.85 | 3.27                     | ± | 0.18 <sup>d</sup>  | 15.26                                   | ± | 1.22 <sup>d</sup> | 25.56                                      | ± | 2.45 <sup>c</sup> | 62.1±1.4 <sup>d</sup>                   |

Results are expressed as mean ± standard deviation. Different letters in the same column indicate significant differences by Tukey test ( $p < 0.05$ ).

*In vitro* digestion simulation of the muffin with the highest TGP content and stevia was performed, as it was the one with the highest bioactive properties. The results in Table 4 show that the BF of the TGP muffin presented no significant differences ( $p > 0.05$ ) with that one of the control muffin (muffin without TGP). In contrast, the CF of TGP muffin presented higher TPC and antioxidant capacity than the control muffin CF, meaning TGP phenolic compounds may be retained by the food matrix during digestion, remaining in the colonic fraction (digestive simulation precipitate) [12]. The results are in

line with Olt et al. [12] on the BF of TGP biscuits, finding differences in the bioactive properties between the CFs of control and TGP biscuits. Further studies of the CF on microbiota effect could state the TGP muffin potential as a functional food. Some authors have stated the potential functionality of other food matrices by studying the effect of the indigestible fraction on microbiota. Mexican lunches [32], Aloe vera polysaccharides [33], chili varieties [34], and guava fruit [35] have shown potential functionality by having a positive effect on microbiota.

**Table 4.** Bioactive properties of muffin with 15% of TGP and 0.85 of stevia after *in vitro* digestion simulation.

| Sample                  |                | TPC<br>(mg GAE/g)      | ABTS<br>( $\mu\text{mol Trolox/g}$ ) | ORAC-FL<br>( $\mu\text{mol Trolox/g}$ ) |
|-------------------------|----------------|------------------------|--------------------------------------|-----------------------------------------|
| Bioaccessible fractions | Control muffin | 2.56±0.27 <sup>a</sup> | 224.4±18.5 <sup>a</sup>              | 152.5±12.8 <sup>a</sup>                 |
|                         | TGP muffin     | 2.70±0.38 <sup>a</sup> | 223.8±15.8 <sup>a</sup>              | 154.7±9.4 <sup>a</sup>                  |
| Colonic fractions       | Control muffin | 3.00±0.13 <sup>a</sup> | 113.5±12.9 <sup>a</sup>              | 114.5±19.8 <sup>a</sup>                 |
|                         | TGP muffin     | 7.23±0.34 <sup>b</sup> | 190.9±10.4 <sup>b</sup>              | 161.2±20.4 <sup>b</sup>                 |

Results are expressed as mean ± standard deviation. Different letters in the same column state significant differences by t test ( $p < 0.05$ ) between groups of samples (bioaccessible fractions separated from colonic fractions).

This study aligns with the Functional Food Science framework by integrating the evaluation of bioactive compounds, food formulation, and digestive bioaccessibility. The persistence of TGP polyphenols in both bioaccessible and colonic fractions demonstrates their potential as functional ingredients contributing to

intestinal antioxidant and anti-inflammatory activity. Mechanistically, these effects are linked to modulation of oxidative stress (ROS) and inflammatory mediators (NO) in colon cell and macrophage models, respectively. The muffin matrix provides a delivery system for phenolics within a fiber-rich environment, highlighting the role of

colonic metabolism and microbiota in phenolic transformation. Such evidence supports the classification of TGP-enriched muffins as sustainable functional foods. Future translational research should focus on human intervention studies and microbiota modulation to validate efficacy and safety.

This study provides novel insights into the valorization of TGP as a functional food ingredient. For the first time, bioaccessible phenolic compounds from TGP were identified, revealing their persistence in both bioaccessible and colonic fractions by demonstrating antioxidant and anti-inflammatory effects in relevant cell models. It introduces the concept of a colonic fraction as a potential functional carrier of phenolics within a solid food matrix (muffin formulation), highlighting an innovative digestion-informed approach to functional food design. These novel findings contribute to the understanding of the stability and biological potential of grape byproduct polyphenols, emphasizing the critical role of the food matrix in modulating their release and activity.

From a practical perspective, the current work demonstrates the feasibility of developing sustainable muffins by incorporating TGP, thereby enhancing nutritional quality. Food manufacturers could use this evidence to optimize formulation levels (up to 15% TGP). Although bioaccessibility in muffins was limited, the colonic fraction represents promising potential to have a health-promoting effect on colon cells and on microbiota, which may influence chronic disease incidence [36]. The findings also may provide future guidance for nutritionists and clinicians in recommending sustainable, phenolic-enriched foods with potential benefits for gut and metabolic health. Overall, the present work results promote the application of the circular economy concept in line with the sustainable development goals. Future *in vivo* studies and sensory analyses should be addressed to consolidate these applications.

## CONCLUSIONS

The present work confirmed for the first time the persistence of phenolic compounds in the bioaccessible fraction of TGP. However, the colonic fraction showed higher antioxidant and  $\alpha$ -glucosidase inhibition than the bioaccessible fraction, meaning most of the phenolic compounds present in TGP may have been retained during digestion. Cell studies showed for the first time that TGP is bioaccessible and has intracellular antioxidant and anti-inflammatory effects in normal human colon cells (CCD-841CoN) and macrophages (RAW264.7), respectively, showing potential to be used as a functional ingredient. Additionally, the feasibility of incorporating TGP into a muffin formulation was demonstrated. After *in vitro* digestion simulation, the bioactive properties of TGP muffin CF showed significant differences with the control muffin CF, meaning TGP phenolic compounds may not have been released from the food matrix during digestion, remaining in the CF.

Future studies of the CF of the muffin with TGP on microbiota effect are needed to state the TGP muffin's potential as a functional food. Overall, TGP demonstrated potential as a functional ingredient through its incorporation into muffins, promoting circular economy strategies, sustainable food production, and the development of functional foods.

**List of Abbreviations:** BF, bioaccessible fraction; CF, colonic fraction; LPS, lipopolysaccharide; NO: nitric oxide; ROS: reactive oxygen species; TGP, Tannat Grape Pomace; TGS, Tannat Grape Skin; TPC: total phenolic content.

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**Authors' Contributions:** AMFF: conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, writing—original draft preparation, writing—review and editing, visualization,

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