Chapter

NUTRITIVE VALUE, TOTAL PHENOLIC CONTENT, AND RADICAL SCAVENGING ACTIVITY BEFORE AND AFTER DIGESTION OF THE LEAVES OF SIX GRAPEVINE (*VITIS VINIFERA* L.) CULTIVARS

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ABSTRACT

In Mediterranean countries, grapevine leaves are an important source of forage for ruminants during the critical period when quality and quantity of pasture herbage is limited. They are also used in human nutrition for the preparation of various typical dishes. The aim of this study was to compare the chemical composition and nutritive value of the leaves of six grapevine (*Vitis vinifera* L.) cultivars (Barbera, Cabernet Sauvignon, Grenache, Nebbiolo, Pinot Noir and Sirah). The same leaves have also been tested to determine the impact of *in vitro* simulated human gastrointestinal digestion on total phenolic content and radical scavenging activity before and after digestion. This study demonstrates that the nutritive value for ruminants and the content of bioactive compounds and related antioxidant capacity for humans depends on the grapevine cultivars. These leaves are rich in bioactive compounds and might provide a significant source of dietary bioaccessible polyphenols with high antioxidant capacity.

Keywords: grapevine, chemical composition, digestibility, fibrous content, gross energy, total phenolic content, radical scavenging activity

INTRODUCTION

Grapes (*Vitis vinifera* L.) are one of the largest fruit crops in the world and in 2017, more than 74 million tons were produced, of which 26 million corresponded to European growers (FAO, 2017). Generally the cultivation of grapevines is aimed at the production of grapes for processing into wine and for fresh consumption, however in Mediterranean countries, shoots and leaves of grapevines are also traditionally browsed by goats and sheep (Heuzé et al., 2017) and proposed for potential use in ruminant feeding (Sanchez et al., 2002; Peiretti et al., 2017). In particular, grapevine leaves are an important source of forage for ruminants during the critical period when the quality and quantity of pasture herbage is limited (Romero et al., 2000; Kamalak, 2005; Gurbuz, 2007). Grapevine leaves can be considered a byproduct of grapes, which have high cell wall content, low energy

value, and low cell wall fraction digestibility, due to the content of lignin and tannins, considered antinutritional factors (Alvira et al., 1983).

In Florida and in several countries of Southern Europe (Greece, Turkey, Bulgaria, Moldova, Romania, Albania, Macedonia, etc.), the Middle East (Iran, Egypt, Lebanon, etc.) and East Asia (Korea, Vietnam, etc.) grapevine leaves are also used for human nutrition in the preparation of various typical dishes including rolls stuffed with rice, meat and spices (Sat et al., 2002; Park et al., 2011; Bekhit et al., 2016; Lima et al., 2016). Stuffed grapevine leaves, in fact, are a typical dish of Arab cuisines that border the Mediterranean basin (Firat and Çetin, 2016; Lima et al., 2017). In general, in the Arabic-speaking countries, these roulades are called *warak enab*, and are used as appetizers, prepared using vine leaves stuffed with a mixture of minced meat, rice, chopped onion, herbs, and spices. Around the world, we find them under different names (*tokat*, *dolmades*, *dolmeh barg mo*, *and Vietnamite loup*) that indicate dishes with a single basic ingredient, grapevine leaves. Their most widespread use is the roll stuffed with meat, rice and spices (famously the *Bulgarian Sarmi* and the *spring rolls* of Albania). Usually these rolls use fresh leaves picked from a plant that has not been treated with anticryptogamic substances. Otherwise, to guarantee their availability all year round, they are salted and stored in containers in a brine solution, blanched and ready to be used. They are a product, sharing properties of fruit and vegetables that could certainly be more included in European diets, which are unlike those found in Middle Eastern countries, where dishes made with grapevine leaves are used daily. Various type of certified organic grapevine leaves are also used in herbal blends as infused teas.

Like fruits, leaves contain beneficial substances, such as organic acids, carbohydrates, stilbenes (resveratrol), vitamins, anthocyanins and tannins (Acquadro et al., 2018). Within the grapevine leaves there are also several enzymatic substances, capable of stimulating biliary secretion. Given the presence of so many beneficial substances, different properties are attributed to these leaves: antioxidants, antiarterosclerotics, cytoprotectives, hepatoprotectives, and cardioprotectives (Bombardelli and

Morazzonni, 1995; Felicio et al., 2001; Bown, 2001; Van Wyke and Wink, 2004; Orhan et al., 2007; Tartaglione et al., 2018).

The aim of this study was to compare the chemical composition and nutritive value of the leaves of six grapevine cultivars (Barbera, Cabernet Sauvignon, Grenache, Nebbiolo, Pinot Noir, and Sirah). Moreover, the impact of *in vitro* simulated human gastrointestinal digestion on total phenolic content and radical scavenging activity before and after digestion have been determined on the same leaves.

MATERIAL AND METHODS

Chemicals and Standards

Reagents for chemical analysis, *in vitro* ruminant digestibility and human simulated gastrointestinal digestion were purchased from Sigma-Aldrich (Milan, Italy). Folin-Ciocalteu's phenol reagent, sodium carbonate (≥ 99.5%), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (97%) (Trolox), and 2,2-diphenyl-1-picrylhydrazyl (95%) (DPPH) were obtained from Sigma-Aldrich (Milan, Italy). Ethanol (≥99.8%) and gallic acid (≥ 98.0%) were obtained from Fluka (Milan, Italy). Ultrapure water was prepared in a Milli-Q filter system (Millipore, Milan, Italy).

Plant Material and Environmental Conditions

The trials were carried out on plots located in an experimental field in the North-West of Italy (45°06′50″N 7°59′13″E) at an altitude of 290 m above sea level. The leaves of six grapevine cultivars (Barbera, Cabernet Sauvignon, Grenache, Nebbiolo, Pinot Noir, and Sirah) were cut in 2017 for each variety, with edging shears. Sampling was only conducted in favourable weather conditions and after the disappearance of dew.

Chemical Analysis

An aliquot of 200 g of each collected leaf sample was used to determine the dry matter (DM), in duplicate, in a forced draft air oven at 105°C overnight. Another aliquot of 200 g was immediately refrigerated, freeze-dried, and then brought to air temperature, ground in a Cyclotec mill (Tecator, Herndon, VA, USA) to pass through a 1-mm sieve, and then stored for analyses performed in duplicate. The samples were analyzed to determine the total nitrogen content (AOAC 1990). Acid detergent fibre (ADF), neutral detergent fibre (NDF) and lignin were determined using an Ankom 200 Fibre Analyser (Ankom Technology Corp., Macedon, NY, USA), according to the method of Van Soest et al., (1991). Gross energy (GE) was determined using an adiabatic calorimeter bomb (IKA C7000, Staufen, Germany).

In Vitro Ruminant Digestibility

The leaf samples were also analysed to determine their *in vitro* apparent digestibility (DMD), using a Daisy II Incubator (Ankom Technology Corp., Fairport, NY, USA), according to Robinson et al., (1999). Freeze-dried samples (0.25 ± 0.01 g) were double-weighed in F57 Ankom bags, with a pore size of 25 µm, heat-sealed and then placed into an incubation jar. Each jar was a glass receptacle with a plastic lid provided with a one-way valve, which prevented the accumulation of fermentation gases, and was filled with 2 L buffered rumen fluid, in anaerobic conditions. Buffer solution was obtained by mixing 266 mL of solution A (KH_2PO_4 10 g/L, $MgSO_4*7H_2O$ 0.5 g/L, NaCl 0.5 g/L, $CaCl_2*2H_2O$ 0.1 g/L, Urea 0.5 g/L) and 1330 mL of solution B (Na_2CO_3 15.0 g/L, Na_2S*9H_2O 1.0 g/L). The jar was then introduced into the incubator. The rumen liquor was collected at a slaughterhouse, from the rumen content of cattle fed a fibre-rich diet (Spanghero et al., 2010). The temperature (39 °C) and agitation were maintained constant and uniform in the controlled chamber by means of continuous rotation. After 48 h of

incubation, the jars were emptied and the bags were rinsed gently. DMD was calculated using the following equation:

DMD (%) = DMwtante- DMwtpost/ DMwtante * 100

where DMwtante is the DM weight before the incubation and DMwtpost is the DM weight after the incubation.

In Vitro Human Simulated Gastrointestinal Digestion

The human simulated gastrointestinal digestion comprising the oral, gastric, and small intestinal steps was performed according to a standardized static *in vitro* method suitable for food described by Minekus et al., (2014). For each stage, digestive juices were prepared for mouth (Simulated Saliva Fluid, SSF), stomach (Simulated Gastric Fluid, SGF) and small intestine (Simulated Duodenal Fluid, SDF). The entire process was performed at 37ºC in a water bath with constant orbital agitation. In the oral phase, one gram of powdered leaves was mixed with 5 mL of SSF containing human salivary α-amylase solution (1500 U/mL). Afterward, the mixture was adjusted to pH 7 ± 0.2 and incubated for 2 min. Then, 10 mL of SGF containing porcine pepsin (2000 U/mL) were added for the gastric phase. The mixture was adjusted to pH 3.0 ± 0.2 and incubated for 2h. Finally, in the duodenal phase 20 ml of SDF containing pancreatin (100 U/mL of trypsin activity) and porcine bile extract (10 mM) were added. The final mixture was adjusted to pH 7 ± 0.2 and samples were incubated for an additional 2 h. The human simulated gastrointestinal digestion was performed in quadruplicate for each sample and ultrapure water was used as the blank. After complete digestion, the pH was adjusted to 5.4 and the samples were immediately transferred to an ice bath to minimize enzyme activity. After centrifugation at 12,500 × *g* at 0°C for 10 min, the supernatants were filtered through a 0.45 μm cellulose acetate membrane filter (VWR, Milan, Italy) and stored at -20ºC for further analysis.

Extraction of Bioactive Compounds from Grapevine Leaves before Human Simulated Gastrointestinal Digestion

To yield the bioactive compounds, 0.25 g of samples were extracted with 5 mL of ethanol-water mixture (50:50, v/v). Extractions were performed at room temperature under constant rotatory oscillation using a VDRL 711 orbital shaker (Asal S.r.l., Milan, Italy) for 2h. The extractions were performed in duplicate for all samples. All extracts were centrifuged at 12,500 × *g* at 0°C for 10 min, and the supernatants were then filtered through a 0.45 μm PTFE filter. Samples were stored at -20 ºC in the dark before analysis.

Total Phenolic Content

The total phenolic content (TPC) of grapevine leaves was assessed according to the Folin–Ciocalteu colorimetric method described by Singleton and Rossi (1965) with some modifications and adapted to a 96-well microplate as described by Barbosa-Pereira et al., (2018). The absorbance was recorded after 1h at 740 nm using a BioTek Synergy HT spectrophotometric multi-detection microplate reader (BioTek Instruments, Milan, Italy). Determinations were performed in triplicate before and after human simulated gastro-intestinal digestion. Quantification was carried out using a standard curve of commercial gallic acid (20-100 mg/L), and the concentration of total phenolic compounds was expressed as mg of GAE/g of dry weight.

Radical Scavenging Activity

The antioxidant capacity of grapevine leaves before and after human simulated gastro-intestinal digestion was determined by the 2,2’-diphenyl-1-picrylhydrazyl (DPPH•) radical scavenging assay described by von

Gadow et al., (1997) with slight modifications and adapted to 96-well microplates as described by Barbosa-Pereira et al., (2018). The decrease in DPPH absorbance was measured at 517 nm using a BioTek Synergy HT spectrophotometric multi-detection microplate reader (BioTek Instruments, Milan, Italy). Determinations were performed in triplicate before and after simulated gastro-intestinal digestion. The inhibition percentage (IP) of the radical DPPH was calculated using the following equation:

$$IP\ (\%) = ((A_0 - A_{30}) / A_0) * 100$$

where A_0 is the absorbance at initial time and A_{30} is the absorbance after 30 minutes. A linear curve of commercial Trolox was used in a range between 12.5 and 300 μM and the results were expressed as μmol of Trolox equivalents (TE)/g of sample.

Statistical Analysis

The variability in chemical composition and in the digestibility of the samples was analysed, to establish its statistical significance, by means of an analysis of variance (ANOVA), using SPSS version 11.5.1 for Windows (SPSS Inc., Chicago, IL, USA) to test the effect of the cultivars. Multiple comparisons of the means were conducted using a post hoc (Tukey test) procedure to establish any differences between cultivars. Differences were considered significant at the $p < 0.01$ level. The total phenolic content and radical scavenging activities of grapevine leaves from different cultivars, undigested and after *in vitro* simulated human gastrointestinal digestion, were compared by variance analysis (ANOVA) with Duncan's post hoc test at the 95% confidence level performed on Statistica version 13.3 software (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Nutritive Value of the Grapevine Leaves

Chemical composition and nutritive value of the grapevine leaves are given in Table 1. The chemical composition of different grapevine cultivars was highly variable, except for DM and crude protein (CP) contents that did not significantly differ and ranged from 31.5 to 37.7% and from 10.8 to 12.0%, respectively. These CP values could be considered sufficient to provide rumen micro-organisms with the required nitrogen content to support their activity. Kok et al., (2007) reported no significant differences in the CP content of grapevine leaves or the summer lateral shoots of four cultivars (Cabernet Sauvignon, Merlot, Sauvignon Blanc and Sémillon) at grape harvest and at two post-harvest dates.

Table 1. Chemical composition (% DM), gross energy (GE, MJ/kg DM), and *in vitro* apparent digestibility (DMD, %) of the leaves of six cultivars of grapevine leaves

	Barbera	Cabernet Sauvignon	Grenache	Nebbiolo	Pinot Noir	Sirah	SEM	*p*
DM (% FM)	37.09 ± 0.04	35.19 ± 2.43	31.48 ± 0.76	37.71 ± 3.04	34.37 ± 4.11	35.80 ± 3.03	0.83	0.337
Crude protein	10.75 ± 0.42	12.01 ± 1.07	11.69 ± 0.06	11.99 ± 0.57	11.12 ± 3.44	11.26 ± 2.09	0.39	0.963
NDF	40.40 ± 0.80^{a}	39.37 ± 1.65^{a}	40.93 ± 0.05^{a}	40.88 ± 0.37^{a}	41.74 ± 0.88^{a}	35.54 ± 0.55^{b}	0.64	0.004
ADF	25.63 ± 0.06^{d}	28.84 ± 1.60^{cd}	29.77 ± 0.90^{bcd}	38.68 ± 0.90^{a}	33.47 ± 0.36^{b}	31.22 ± 1.72^{bc}	1.25	0.001
Lignin	6.28 ± 0.37^{b}	6.66 ± 0.45^{b}	5.88 ± 0.46^{b}	7.45 ± 0.33^{ab}	8.44 ± 0.49^{a}	7.55 ± 0.52^{ab}	0.28	0.009
GE	17.67 ± 0.34	17.92 ± 0.49	17.76 ± 0.66	17.90 ± 0.84	18.13 ± 0.08	17.77 ± 0.66	0.13	0.968
DMD	60.70 ± 0.92^{ab}	61.17 ± 0.25^{ab}	62.83 ± 1.00^{a}	53.49 ± 0.93^{c}	49.27 ± 1.17^{d}	58.60 ± 1.30^{b}	1.46	0.001

[abcd] Values with different letters within a row differ for $p < 0.01$ (final column). SEM = standard error of mean.

As far as potential nutritive value of leaves for ruminants is concerned, Gurbuz (2007) evaluated the chemical composition, *in vitro* gas production and *in situ* DM and CP degradation of leaves of four grapevine varieties (Kabarcik, Mahrabasi, Kibris and Ak) cultivated in Turkey. This author found that CP content of Kabarcik leaves was significantly higher than that of other varieties and CP contents of leaves ranged from 9.94 to 12.14%, with values similar to our results. On the other hand, Romero et al., (2000) and Rebolé (1994) found a lower CP content in grapevine cultivars than that found in the present study, because their samples also contained branches in addition to the leaves. Romero et al., (2000) reported a CP content of 6.8% for grapevine leaves and 7.3% for hay and concluded that the low digestibility of the grapevine leaves might be due to factors such as the low CP digestibility and high content of lignin and condensed tannins. Rebolé (1994) found a CP content of 5.0% for ensiled and 6.0% for fresh grapevine branches. Kamalak (2005) determined the nutritive value of leaves of ten varieties of grapevine grown in Turkey and found that their CP content ranged from 7.9 to 11.2%, while the *in vitro* DMD ranged from 59.8 to 75.3% and digestibility was negatively correlated with cell wall and condensed tannin contents. The varieties with highest protein content and *in vitro* DMD were Sultani, Perlette, and Honusu, which have the potential to be good quality forages for ruminants during the critical periods.

As far as the content of fibrous components is concerned, the NDF content reported in Table 1 was generally high (39.4÷41.7%), except for leaves of Sirah (35.5%), while ADF content significantly differed and ranged from 25.6% for Barbera leaves to 38.7% for Nebbiolo leaves. Our results were higher than those reported by Gurbuz (2007), where NDF and ADF content of four varieties of grapevine leaves ranged from 28.5 to 38.4% and 18.8 to 28.4%, respectively, comparable to those reported by Romero et al., (2000).

In our study GE content did not significantly differ (from 17.7 to 18.1 MJ/kg DM), while digestibility results were influenced by fibrous components and in particular by ADF content, as reported by Romero

et al., (2000). In particular, we found that leaves of Pinot Noir, Nebbiolo and Sirah, with the highest ADF and lignin content, were the least digestible cultivars, while the other three cultivars were more digestible due to their low content of ADF and lignin in their leaves. Gurbuz (2007) found significant differences among the four varieties of grapevine in terms of gas production at all incubation times. The Kabarcik cultivar showed a gas production higher than that reported for the other three varieties. This author concluded that leaves of Kabarcık might have a higher potential nutritive value for sheep in terms of rumen and whole tract digestion, because these leaves had low NDF, ADF and condensed tannins and high CP contents, with a higher rank value in terms of DM disappearance and CP degradation.

There are some studies on grapevine pruning residues that, in addition to the branches, also contain numerous leaves. Rebolé and Alvira (1986) found a CP content of 6.7% in prunings with leaves of grapevine cv. Valdepeñas that ranged from 5.8 to 6.8% when ensiled using different additives. *In vitro* digestibility was 42.7% and 32.9% for fresh and silage, respectively. Peiretti et al., (2017) reported the differences in chemical composition, GE, *in vitro* DMD and fatty acid profile of green pruning residues of the same six grapevine cultivars studied in the present paper. They found significant differences among cultivars in terms of ash, NDF, ADF, and lignin contents, which ranged from 5.4 to 7.0%, 49.5 to 54.2%, 37.5 to 42.5%, and 9.1 to 14.2%, respectively. Moreover, the NDF, ADF, and lignin contents of the green pruning residues were negatively correlated to the *in vitro* DMD, which ranged from 44.9 to 54.4%. The higher cell wall contents and lower digestibility values make the green pruning residues less nutritive to ruminants than the leaves alone.

Winkler et al., (2015) determined the chemical composition and nutritive value of dried and ensiled white and red grape pomace cultivars originating from Germany. Grape pomace from red cultivars (Dornfelder, Pinot Noir and Portugais Bleu) showed higher contents of organic matter, CP, ether extract and crude fibre. Concentrations of sugar and ash were higher in white cultivars (Pinot blanc and Riesling). The digestibility of organic matter, CP, ether extract and crude fibre and the energy content

were lower for dried white than for dried red grape pomace, whereas the digestibility of cell walls was higher for dried white than dried red grape pomace.

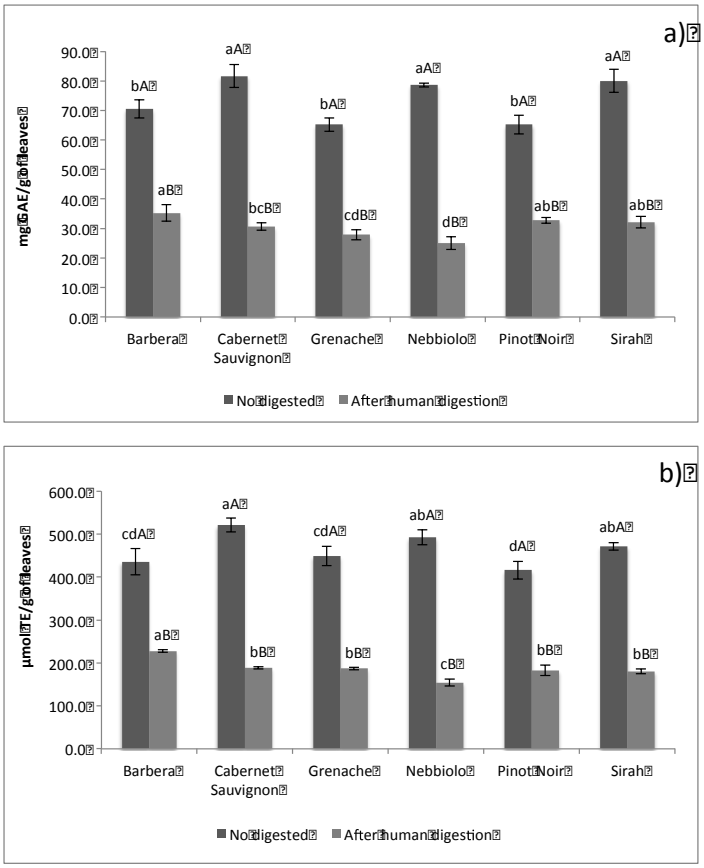

Figure 1. (a) Total phenolic content (mg GAE/g) and (b) radical scavenging activity (µmol TE/g) of six cultivars of grapevine leaves analysed before (not digested) and after *in vitro* simulated human gastrointestinal digestion. Values are expressed as mean ± standard deviation. Capital letters indicate significant differences ($p < 0.001$) between not digested samples and samples taken after *in vitro* simulated human gastrointestinal digestion. Lower case letters indicate significant differences ($p < 0.05$) between the samples of the six cultivars of grapevine leaves (GAE: Gallic acid equivalent. TE: Trolox equivalent).

Effect of Human Simulated Gastrointestinal Digestion on the Bioaccessibility and Antioxidant Capacity of Bioactive Compounds in Grapevine Leaves

The impact of *in vitro* simulated human gastrointestinal digestion on TPC and the DPPH radical scavenging activity (RSA) of the several grapevine leaves are shown in Figure 1. The TPC of the six varietals analysed before simulated gastrointestinal digestion (not digested) ranged from 65.2 mg GAE/g to 81.6 mg GAE/g of leaves (Figure 1a). The highest contents in bioactive compounds were found in Cabenet Sauvignon, Sirah and Nebbiolo leaves, while Grenache, Pinot Noir and Barbera leaves showed the lowest amounts of polyphenols among the varietals analysed. These TPC values are double those described by Dinis et al., (2016) for the Touriga Nacional varietal (around 40 mg GAE/g DW) and similar to that found by Pantelić et al., (2017) in the Cabernet Franc varietal (76.0 g GAE/kg DW), which are different varietals from those used in the present study. After complete gastrointestinal digestion, the bioaccessibility of the phenolic compounds in grapevine leaves ranged between 31.0% and 50.3% depending on the varietal. The decrease in TPC was found to be higher in samples from Nebbiolo (25.1 mg GAE/g) and Cabernet Sauvignon (30.7 mg GAE/g) with losses of bioactive compounds of 68.1 and 62.5%, respectively. On the other hand, Pinot Noir (32.4 mg GAE/g) and Barbera (35.2 mg GAE/g) cultivars showed lower degradation of polyphenols due to enzymatic digestion. Therefore, for these varietals, the bioaccessibility of bioactive compounds to be absorbed in the small intestine was higher than 50%. Gunathilake et al., (2018) described higher reduction of TPC values (up to 85%) after gastrointestinal digestion than that observed in the present study.

The antioxidant capacities of the six varietals analysed before and after simulated gastrointestinal digestion are shown in Figure 1.b. The high initial RSA values of samples before the digestion process ranged from 626.11 to 521.06 μmol TE/g of sample. As for TPC, the leaves from Cabernet Sauvignon, Sirah and Nebbiolo varietals showed high antioxidant capacity, while Pinot Noir showed lower RSA values. These RSA values

are similar to those described by Pantelić et al., (2017) for Cabernet Franc and Pinot Gris varietals with RSA values of 867 and 429 µmol TE/kg DW respectively. A reduction in the antioxidant capacity up to 60% was observed in samples after gastrointestinal digestion with RSA values ranging from 154.3 to 227.8 µmol TE/g of leaves. Barbera samples underwent lower losses of antioxidant capacity of 47.7%, while for Nebbiolo leaves the reduction of RSA values after digestion was 68.7%. After complete digestion, Barbera became the varietal with highest antioxidant capacity and the Pinot Noir the one with lowest activity. The other varietals displayed similar RSA values around 185 µmol TE/g. Although the digestion process reduces the antioxidant capacity of samples, the RSA values were still higher when compared with other grapevine leaves after digestion (Gunathilake et al., 2018). The antioxidant capacity of grapevine leaves analysed in this study was strongly correlated with the total phenolic content describe above ($r = 0.992$) and therefore this activity could be related to the presence of polyphenols. These data highlight the potential use of these leaves as a good source of natural antioxidants that could contribute to several health benefits to humans. These results, therefore, might encourage the consumption of grapevine leaves as a food ingredient.

CONCLUSION

The present study demonstrates that the nutritive value for ruminants, content of bioactive compounds and related antioxidant capacity depends on the grapevine varietal. These leaves are rich in bioactive compounds and might provide a significant source of dietary bioaccessible polyphenols with high antioxidant capacity for humans and animals.

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