STUDY OF THE ANTIMICROBIAL ACTIVITY OF WINEMAKING BY-PRODUCTS AGAINST ANTIBIOTIC RESISTANT COMMENSAL BACTERIA OF ANIMAL INTESTINAL ORIGIN

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Introduction

In the context of circular economy, wineries generate large amounts of grape pomaces whose efficient exploitation is currently an important challenge, and they may become suitable candidates for developing viable and up-graded food and feed products. Grape pomace is estimated to represent around 13 % of the total grape weight used in the winemaking process, and it means that 612,000 tonnes were generated in Spain in 2021 (OIV, 2022). Grape pomace can be used to obtain ethanol and grape seed oil; nevertheless, phenolic compounds still remain in the waste product. These grape phenolic compounds are secondary metabolites synthesized by the plant cells to act as natural defences against ultraviolet radiation or aggression by pathogens. The antioxidant properties of these phenolic compounds and their benefits for human health have been demonstrated (Ferrer-Gallego and Silva, 2022). However, currently no systematic study has been reported on their activity against the growth of antibiotic-resistant commensal bacteria that are found in the intestine of humans and animals. These resistant bacterial strains are carriers of antibiotic resistance genes, which can be disseminated to other commensal and pathogenic bacteria.

Escherichia coli is an important Gram-negative species of saprophytic bacteria of the intestinal microbiota of humans and animals; nevertheless, they may act as opportunistic pathogens under certain circumstances and may develop problematic antibiotic resistances that hinder treatments against bacterial infections. *E. coli* is as well an indicator microorganism of faecal contamination, antibiotic resistance and dissemination through the food chain (Sanz et al., 2021). Among the numerous properties reported for phenolic compounds, antibacterial activity is included (Álvarez-Martínez et al., 2020). Few studies have been reported on the effect on the growth of antibiotic resistant bacteria, among them phenolic extracts from fresh grape skins and seeds have been shown to possess effect on their growth (Fernández-Pérez et al. 2018, 2020).

The objective of this study was to analyse the chemical composition of six grape phenolic extracts, obtained from grape pomaces generated as winery by-products, and to evaluate their antibacterial activity against susceptible and multidrug resistant *E. coli* strains with the phenotype of extended spectrum beta-lactamases.

Material and methods

1. *Bacterial strains*

All strains included in this study (Table 1) were of intestinal origin and were isolated from healthy animals. They belonged to *E. coli* species and two of them were multidrug resistant and ESBLs (extended-spectrum beta-lactamases) producers.

Table 1: Genotypic and phenotypic characterisation of the antimicrobial profiles of the *Escherichia coli* strains included in this study.

Name	Species	Phenotype of resistance to antibiotics	Resistance genes	Origin
C7023	*Escherichia coli*	susceptible	none	cow
C7067	*Escherichia coli*	susceptible	none	rabbit
C7577	*Escherichia coli*	AMP, CAZ, CTX, NAX, TET, SXT	SHV-12 (ESBL)	roe deer
C6840	*Escherichia coli*	AMP, CTX, NAX, TET, SXT	CTX-M-14a (ESBL)	coati

AMP (ampicillin); CAZ (ceftazidime); CTX (cefotaxime); NAL (nalidixic acid); TET (tetracycline); SXT (sulfamethoxazole-trimethoprim) ; ESBL (Extended-Spectrum β-Lactamase)

2. *Grape phenolic extracts*

A total of six extracts shown in Table 2 were studied. T and M extracts were obtained at laboratory scale from pomaces of red grape vinifications of Vitis Vinifera L. cv. Tempranillo (2019 vintage in D.O.Ca. Rioja, Spain) and Merlot (2013 vintage in D.O. Ribera del Duero, Spain) respectively. Vinifications were conducted following the traditional method, i.e. fermentations took place in presence of the crushed red grapes, seeds and stems. Pomaces were frozen and stored at -20 ºC until the extraction. These monovarietal T and M extracts were obtained by maceration with the pectolytic product Lallzyme EXV (Lallemand Inc.), homogenization in the Ultraturrax (IKA Werke GmbH & Co. KG. Germany) high shear dispersing machine, and conventional ethanolic extraction with sonication (14 kHz, 20 min). The obtained monovarietal extracts were dried in a laboratory centrifugal evaporator with cold trap Jouan RCT-60. Four commercial natural food and feed supplements of red and white grape phenolic extracts (generously provided by Alvinesa Natural Ingredients, Daimiel, Ciudad Real, Spain) were included in this study as well. These dry phenolic extracts from Alvinesa Natural Ingredients are entirely constituted by phenolic compounds (premium selected blending of monomers, dimers, oligomers, and polymers) from grapes. These extracts are currently approved by the EFSA either for human (55705, 55709 and 55710) or animal (55707) consumption.

Table 2: Grape phenolic dry extracts included in this study

Extract	Description
T	Extract of Tempranillo red grape pomace
M	Extract of Merlot red grape pomace
	Commercial phenolic extracts
55705	Tannins extracted from white grape skins
55707	Extract from red and white grape pomaces
55709	Extract from grape pomaces
55710	Extract from selected fresh red pomaces

*Extract commercial names: 55705. VinteraTM White grape skin tannins; 55707. VinteraTM Dry grape extract - Animal feed; 55709. VinteraTM Grape extract ≥ 50% Polyphenols; 55710. VinteraTM Grape skin extract - EV12 powder

3. *Chemical analysis of the extracts*

Chemical analyses of the six extracts were performed by the Service of Instrumental Analysis of the Institute of Science of Vine and Wine (ICVV, Logroño, Spain). Phenolic profiles were obtained using an UHPLC (ultra-high performance liquid chromatography) chromatograph (Shimadzu Nexera) equipped with a triple quadrupole/ion trap mass spectrometer ABSciex 3200QTRAP®, and an atmospheric pressure ionization source (ESI). A Waters Acquity UPLC BEH C18 (100 mm x 2.1 mm, 1.7 mm) column was used. Data processing was performed with the software package Analyst® 1.6.2 and MultiQuant® 3.0.2 (AB Sciex, USA). Chemical profiles of the phenolic extracts included 32 phenolic compounds, which were grouped into eight families: flavonols, anthocyans, hydroxybenzoic acids, hydroxycinnamic acids, flavan-3 ols, procyanidins, stilbenes and lignans.

4. *Antibacterial activity*

The potential antibacterial activity of the phenolic extracts of our study was tested by the microtiter dilution assay method (Rojo-Bezares et al. 2007) against the susceptible and multidrug resistant *E. coli* strains shown in Table 1, and minimal inhibitory concentration (MIC) values were determined. Briefly, double serial dilutions of the phenolic extracts in water were prepared in the concentration range from 6.3 to 0.2 mg/ml for T and M extracts, and from 8 to 0.13 mg/ml for the commercial extracts, in concordance with their solubility in water. They were tested in bacterial cultures in BHI broth at 30 ºC in the Tecan Spark® 10M multimode microplate reader for 24 h. Optical density at 600 nm was measured to determine bacterial growth. Experiments were performed in triplicate, and positive and negative controls were included in all the assays. Bactericide activity was determined by subculturing the samples for 48 h onto BHI-agar plates without the tested extract, and minimal bactericide concentration was defined as the minimal concentration of the antimicrobial agent that had killed more than 99.9% of the initial inoculum after incubation for 48 onto BHI-agar plates.

5. *Statistical analysis*

Statistical analysis of the results obtained in triplicate was performed to determine mean values, standard deviations, and significant differences. Principal component analysis (PCA), analysis of variance (ANOVA) and post-hoc analysis using Tukey's test at a significance level of p=0.05 were used. IBM-SPSS Statistics 22.0 for Windows (IBM-SPSS Inc., Chicago, IL, USA) was used for data processing.

Results and Discussion

1. *Phenolic composition of the extracts*

Chemical analysis by UHPLC of the six studied extracts identified 32 phenolic compounds that were grouped into the following phenolic families: hydroxycinnamic acids, hydroxybenzoic acids, flavonols, flavan 3-ols, procyanidins, stilbenes, lignans and anthocyans (Table 3).

Table 3. Phenolic composition, classified by families, of the six extracts (ng/mg).

extract	55705	55707	55709	55710	M	T
Hydroxycinnamic acids	5037.19 ± 122.12a	1812.89 ± 66.81b	329.24 ± 8.47c	4608.98 ± 43.52d	191.71 ± 7.85c	204.82 ± 7.14c
Hydroxybenzoic acids	1433.89 ± 21.31a	1026.57 ± 8.38b	946.70 ± 9.33c	1805.95 ± 33.13d	1573.33 ± 7.55e	689.88 ± 10.15f
Flavonols	46935.89 ± 2484.93a	4705.25 ± 200.75b	538.89 ± 14.18c	17522.35 ± 702.46d	1537.11 ± 50.29c	711.84 ± 24.00c
Flavan 3-ols	23647.10 ± 384.14a	35447.46 ± 756.13b	11187.52 ± 223.01c	20907.48 ± 1041.85d	572.45 ± 15.25e	26.50 ± 1.99e
Procyanidins	9164.50 ± 211.40a	67295.48 ± 2339.26b	13849.50 ± 634.99c	15585.73 ± 357.69c	348.47 ± 9.13d	23.65 ± 0.89d
Stilbenes	23.28 ± 1.76a	56.00 ± 2.62b	5.88 ± 0.31c	305.35 ± 2.87d	13.29 ± 0.42a	22.69 ± 0.46e
Lignans	60.71 ± 2.25a	290.44 ± 12.86b	278.71 ± 18.41b	23.38 ± 2.14c	6.35 ± 0.60c	2.50 ± 0.42c
Anthocyans	161.56 ± 5.11a	6300.02 ± 183.69b	2.30 ± 0.28a	24279.74 ± 730.75c	12701.12 ± 185.09d	5238.57 ± 102.19e
TOTAL phenolic content	86464.12 ± 3166.28a	116934.11 ± 2355.25b	27138.73 ± 823.88c	85038.96 ± 2800.13a	16943.83 ± 192.01e	6920.46 ± 119.55f

Values are expressed as means ± SD. Different letters indicate significant statistical differences ($p \leq 0.05$)

Table 3 shows that the highest total phenolic content was that of the commercial extract 55707, which showed as well the highest contents of procyanidins and flavan 3-ols. The extract 55710 showed the highest contents of hydroxybezoic acids, stilbenes and anthocyans, as expected from an extract obtained from selected fresh red pomaces. Both M and T monovarietal extracts obtained at laboratory scale reached lower phenolic contents than the commercial extracts, and the M extract from Merlot pomaces showed higher total phenolic content than the T extract from Tempranillo pomaces, which is in agreement with other studies that reported slightly

higher colour intensity and phenolic content of grapes and wines of Merlot than of Tempranillo variety (Lasanta et al., 2023). This result highlights the reported high diversity among different varieties of the *Vitis vinifera* species (Bigard et al., 2018), which is a globally cultivated crop in all the continents with the exception of Antarctica, and which still maintains consistent fruit trait diversity despite the high genetic pressure performed on it.

Figure 1 shows the outcome of the principal component analysis of the phenolic composition data, whose variables were the eight phenolic families. This analysis revealed that 89 % of the variance (Function 1) was related to the following families, indicated in order of relevance: flavan 3-ols, procyanidins, anthocyans and stilbenes. The richest phenolic extracts were located on the positive side of the first component axis, standing out the extract 55707 with the highest contents of procyanidins and flavan 3-ols, followed by extract 55710, which showed the highest contents of anthocyans and stilbenes. In the middle region, located near the origin of the cartesian plot, were located the extracts with intermediate contents of the phenolic families, and on the negative side were located both M and T monovarietal extracts obtained by conventional ethanolic extraction at laboratory scale.

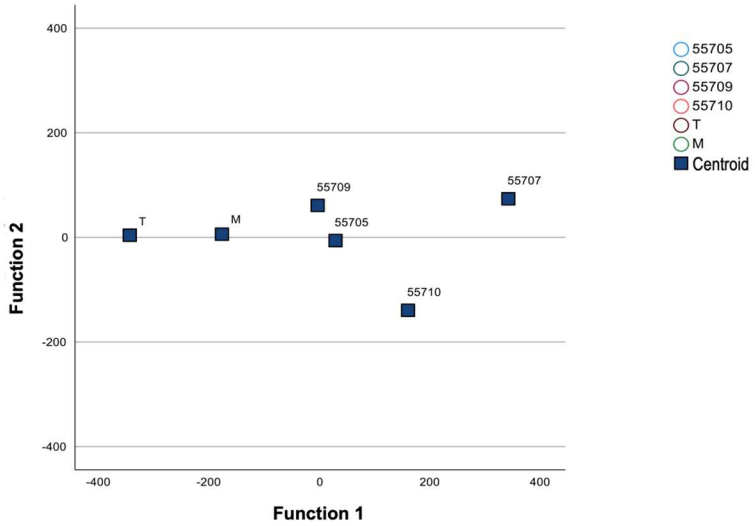

Figure 1: PCA results of the phenolic composition by families. Squares indicate centromeres, which are positioned over the corresponding triplicate sample points (hidden under the square marker) for each of the studied phenolic extract.

2. *Antibacterial activity of the phenolic extracts*

All the phenolic extracts of our study showed antibacterial activity against the intestinal *E. coli* strains of animal origin that were tested: two multidrug resistant *E. coli* strains (C6840 and C7577) and two antibiotic susceptible *E. coli* strains (C7067 and C7023). Figures 2 and 3 show the resulting growth curves of the antibacterial assays of each of the phenolic extracts against the resistant strain C6840. These are representative growth curves of the assays that were obtained against the four *E. coli* strains of our study. A direct correlation between the concentration of the phenolic extract in the culture broth and the decrease of bacterial growth was evidenced by the results in all the assays.

It should be noted that the observed effect was bacteriostatic in all cases, and no bactericidal activity was detected in the studied range of extract concentrations, as bacterial growth was recovered after removing the phenolic extract from the culture broth and subculturing the sample for 48 h. Gram-negative bacteria possess an additional outer membrane containing lipopolysaccharides that covers the bacterial cell wall (Silhavy et al., 2010), therefore they can show higher resistance to antimicrobial agents that should reach the cell wall and the membrane to exert their bactericidal activity. This could be the case of the *E. coli* strains of our

study, which were inhibited in their growth in presence of the phenolic extracts, but recovered after removing the active polyphenols from the culture broth.

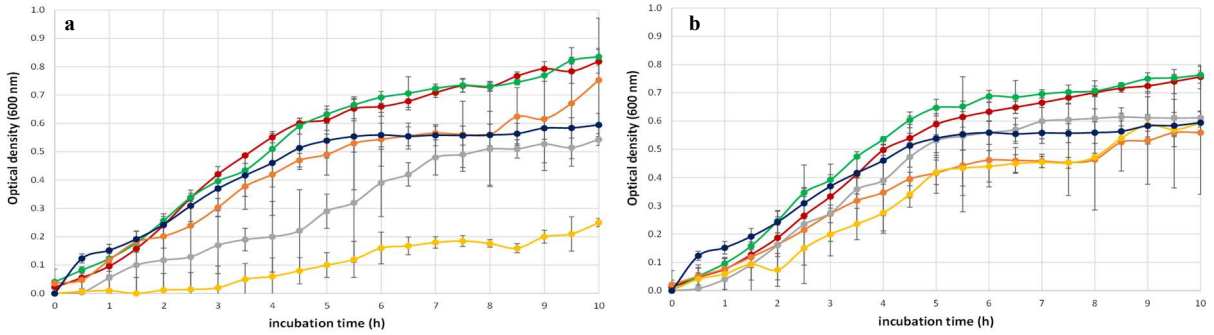

Figure 2. Growth curves of the resistant strain C6840 in presence of (a) M extract, and (b) T extract, in the following concentrations (mg/ml): 0 (red): 0.2 (green); 0.4 (blue); 0.8 (orange); 1.6 (grey) and 3.2 (yellow). Each dot represents the mean value of triplicates and bars represent the corresponding standard deviation.

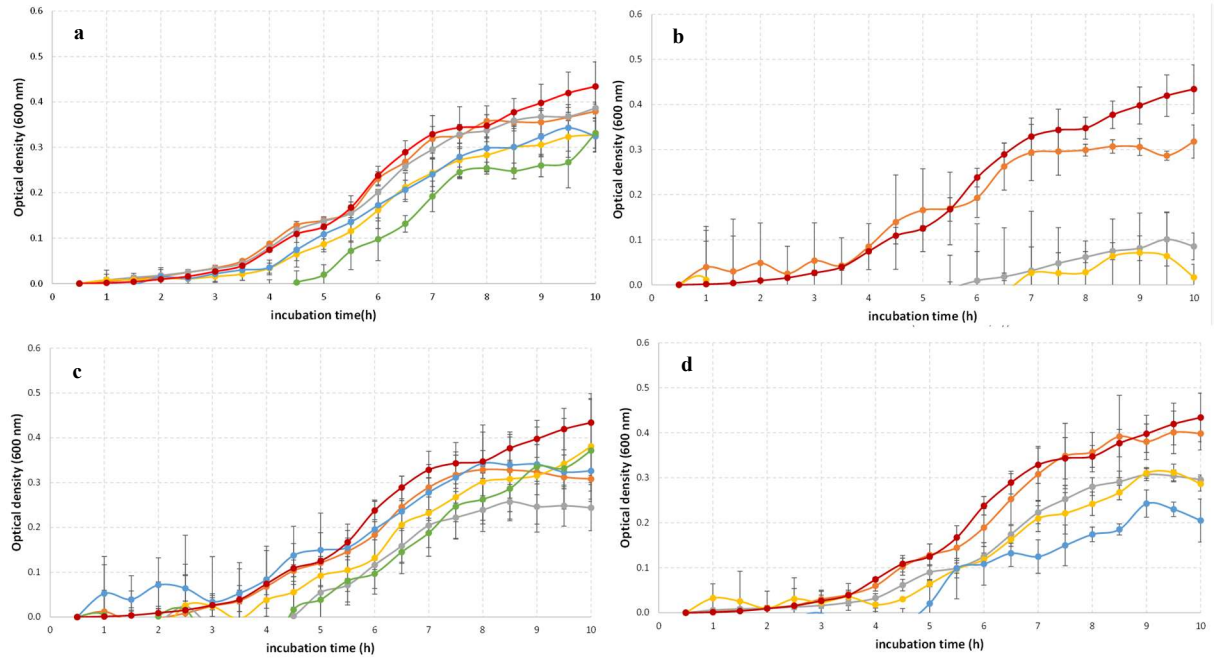

Figure 3. Growth curves of the resistant strain C6840 in presence of (a) extract 55705, (b) extract 55707, (c) extract 55709, and (d) extract 55710, in the following concentrations (mg/ml): 0 (red); 0.13 (orange); 0.25 (grey); 0.5 (yellow); 1 (blue) and 2 (green). Each dot represents the mean value of triplicates and bars represent the corresponding standard deviation.

Table 4 shows the MIC values obtained for each phenolic extract against our collection of *E. coli* strains. Results were slightly different depending on the assayed phenolic extract. Thus, extract 55710 showed higher antibacterial activity than the other extracts, followed by extract 55707, and both extracts were especially active against the resistant *E. coli* strains. These antibacterial activities correlated with the results of phenolic composition in that extract 55707 contained the highest total amount of phenolic compounds, it comprised the highest contents of flavan 3-ols and procyanidins, and both phenolic families were discriminating variables in the PCA. Extract 55710 followed in total phenolic content and showed the highest contents of anthocyans and stilbenes, both discriminating variables that followed flavan 3-ols and procyanidins in relevance in the PCA.

Table 4. Minimal inhibitory concentration (MIC) values (mg/ml) of each extract against the collection of *E. coli* strains.

	***E. coli* strains**			
Phenolic extract	C6840 (R)	C7577 (R)	C7067 (S)	C7023 (S)
55705	4.0	4.0	4.0	4.0
55707	1.0	4.0	4.0	4.0
55709	4.0	4.0	4.0	1.0
55710	2.0	1.0	4.0	4.0
M	6.3	6.3	-	-
T	6.3	3.2	-	-

R: antibiotic resistant bacteria; S: antibiotic susceptible bacteria; -: not analysed.

These results are in agreement with previous studies of our group that reported inhibitory activity of Tempranillo skin extracts from fresh grapes (MIC 6.3 mg/mL) against *E. coli* strains (Fernández-Pérez et al., 2018). Our results are also in accordance with studies on the activity of polyphenols against *E. coli* strains, that reported MIC values in the range of 6-50 mg/mL for flavan-3-ols and slightly higher values, in the range of 10-100 mg/mL, for anthocyans from a variety of vegetable sources (Ma et al., 2019).

In summary, the overall results of this work show that the studied phenolic extracts exert an inhibitory effect on the bacterial growth of intestinal *E. coli* strains, both sensible and resistant to antibiotics, and that it is not a bactericide effect. In addition, it is shown that grape pomaces are rich sources of bioactive phenolic extracts, and they can become important feedstocks for additives and other upgraded products of interest in animal farming.

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