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Grape Phenolic Extract Potentially Useful in the Control of Antibiotic Resistant Strains of *Campylobacter*

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ABSTRACT

In this work, a grape phenolic extract obtained by methanol extraction has been demonstrated to be effective in inhibiting the growth of different strains and species of *Campylobacter*, one of the most important bacterial food-borne pathogens causing gastroenteritis worldwide. Noteworthily, it was particularly effective against several strains presenting multiple antibiotic resistances. In all cases, the minimum inhibitory concentration (MIC) was lower than 300 mg GAE/L, being of 60 mg GAE/L for one of the most resistant strains (*C. coli* LP2), while the others were between 120 mg GAE/L and 180 mg GAE/L. The analytical study of the main phenolic compounds in the grape extract revealed that it was mainly constituted by catechins (85.7%) and phenolic acids (13.7%). However, experiments developed using pure standards demonstrate that phenolic acids (such as gallic, p-hidroxibenzoic, vanillic, and homovanillic acids) were the most active, provoking a *Campylobacter* growth decrease between 6.7 and 7.6 log, while epicatechin was the only catechin with activity as pure compound (1 log of growth decrease).

KEYWORDS

***Campylobacter*; Food-Borne Pathogen; Antibiotic Resistance; Grape Phenolic Extract; Phenolic Acids; Flavanols**

1. Introduction

Campylobacter species are the leading causes of bacterial food-borne gastroenteritis worldwide and the species *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter* coli (*C. coli*) cause more than 95% of the infections attributed to this genus [1]. *Campylobacter* infections in humans are usually characterized by self-limiting diarrhea, abdominal cramps, nausea and fever, but severe neurological sequelae, bacteremia, and other extraintestinal complications may develop less frequently [2]. Several sources of *Campylobacter* infection in humans have been suggested, but the most common is mainly associated with the consumption and/or handling of poultry meat, especially fresh broiler meat [3]. Although most infections are resolved without specific treatment, antimicrobial therapy can be critical in invasive or severe infections. Fluoroquinolone agents, like ciprofloxacin and macrolides such as erythromycin, are commonly used for the treatment of infections caused by *Campylobacter* [4]. However, the rise in the incidence of infections caused by antibiotic-resistant strains of *Campylobacter* makes this illness increasingly difficult to treat [5]. Moreover, since a large proportion of the European Union (EU) chicken production is contaminated with the pathogen [6] and given the recent ban by the European Union on the use of antibiotics in animal feed to promote growth [7], it is essential to search for new, natural and sustainable strategies to reduce the incidence of *Campylobacter* in the food chain, especially in its main host. Consumer concerns about the

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safety of food have increased, and in this regard, there is a growing interest in the use of natural antibacterial compounds, like plant extracts rich in phenolic compounds, as food preservatives. In the last years, different works about the antimicrobial properties of wine and grape phenolic compounds have been published, and several studies have shown that these compounds could inhibit the growth of different food-borne bacteria [8,9]. Concerning *Campylobacter*, other researchers have reported that some phenolic compounds from grape leaves can have antimicrobial activity against this pathogen [10], contributing to modulating the resistance to macrolide antibiotics [11]. We have previously reported the antimicrobial activity of a commercial extract of GSE against *Campylobacter*, identifying the main phenolic compounds related with the behavior observed [12]. In this work, we have obtained several grape extracts using two solvents (methanol and water). The most active extract has been selected to study its antibacterial activity against different species and strains of *Campylobacter*, identifying the main compounds responsible for the antibacterial activity. The bactericidal effect was compared to that of 10 different antibiotics, with the purpose of establishing the potential of grape phenolic compounds in the control of *Campylobacter*.

2. Materials and Methods

2.1. Bacterial Strains, Growth Media, and Culture Conditions

The microorganisms used in this study included 12 different strains: 9 of *C. jejuni* and 3 of *C. coli*. Strain specification and origin of the specimen is provided in **Table 1**.

Table 1. Source of *Campylobacter* species obtained from veterinary, clinical and collection libraries. La Paz and Carlos III are hospitals of Madrid, Spain.

Specie	Strain	Origin	Source
Campylobacter jejuni	LP1	Clinical	La Paz
	11168	Collection	NCTC[a]
	118	Clinical	Carlos III
	11351	Collection	NCTC
	CIII	Clinical	Carlos III
	CN1	Veterinary	CIAL[b]
	CNL1	Veterinary	CIAL
	CNL2	Veterinary	CIAL
	7572	Collection	CECT[c]
Campylobacter coli	LP2	Clinical	La Paz
	CNL4	Veterinary	CIAL
	7571	Collection	CECT

[a]Bacterial cultures obtained from the National Collection of Types Cultures (NCTC), UK, [b]Collection from Instituto de Investigación en Ciencias de la Alimentación, (CIAL), [c]Spanish Collection of Type Cultures (CECT).

All strains were stored at −80˚C. Liquid growth medium for *Campylobacter* strains consisted of Brucella Broth (BB) (Becton, Dickinson, & Company, New Jersey, USA). The agar plating medium consisted of Müeller-Hinton agar supplemented with 5% defibrinated sheep blood (MHB) (Becton, Dickinson, & Company). The frozen strains were reactivated by inoculation in MHB and incubation under microaerophilic conditions (85% N_2, 10% CO_2, 5% O_2) using a Variable Atmosphere Incubator (VAIN) (MACS-VA500) (Don Whitley Scientific, Shipley, UK) at 42˚C for 48 h. Isolated colonies were inoculated into 50 ml of BB and incubated under stirring at 130 rpm on an orbital shaker (Shaker S3) (Elmi, Riga, Latvia) at 42˚C for 24 h in microaerophilic conditions in the VAIN. These bacterial inocula cultures (~1 × 108 CFU/ml) were used for the antibacterial activity assays.

2.2. Antibiotic Susceptibility Testing

The antibiotic susceptibility was assessed for each strain following the Kirby-Bauer disc diffusion method based on the performance standards for antimicrobial disk susceptibility test [13]. The bacterial inoculum in BB was spread onto MHB using sterile cotton-tipped swabs. Antimicrobial discs (Oxoid, Basingstoke, UK) were placed on the inoculated MHB plates and they were incubated in the VAIN for 48 h. The following antimicrobial discs were used: aztreonam, tetracycline, gentamicin, cephalothin, amoxicillin-clavulanic acid, nalidixic acid, chloramphenicol, erythromycin (all 30 µg), streptomycin (25 µg), and ciprofloxacin (5 µg). Interpretation of the results was performed using the resistance breakpoint for *Campylobacter*s described by others [14,15]. When no breakpoints were available for *Campylobacter*s, the resistance breakpoint described by CLSI for Enterobacteriaceae was used. The breakpoints are shown in **Table 2**.

2.3. Grape Extracts Preparation

Phenolic extracts were prepared from three varieties of *Vitis vinifiera* grapes: Tempranillo, Garnacha and Cabernet Sauvignon. Extraction and concentration of the phenolic fraction was carried out by the procedure described by Pallauf *et al.* [16]. Two different solvents were used in the extraction process: methanol, as non-polar solvent, and water (polar solvent).

Briefly, fresh grapes were homogenized using an Ultra-Turrax T25 (IKA-WERKE GmbH & Co., Staufen, Germany) for 2 - 3 min. to obtain 100 g of grape's homogenated. 100 ml of methanol 100% (Sigma-Aldrich, Missouri, USA) or water was added to the homogenate and mixed for 15 min. Afterwards, it was centrifuged for 10 min. at 4500 rpm and the supernatant was collected. The extraction process was repeated twice more.

Table 2. Breakpoints for *Campylobacter* spp.

Antibiotic	Potency	R	I	S
Gentamicin**	10 µg	≤12	13 - 14	≥15
Cephalotin***	30 µg	≤14	15 - 17	≥18
Streptomycin***	10 µg	≤10	11 - 12	≥15
Tetracyclin**	30 µg	≤14	15 - 18	≥19
Cloramphenicol*	30 µg	≤11	12 - 22	≥23
Eritromycin*	15 µg	≤15	16 - 18	≥19
Aztreonam***	30 µg	≤15	16 - 21	≥22
Nalidixic Acid**	30 µg	≤13	14 - 18	≥19
Ciprofloxacin*	20/10 µg	≤13	14 - 17	≥18
Amoxicillin Clavulanic Acid***	5 µg	≤18	19 - 23	≥24

*Miflin *et al.* (2007), **Luangtongkum *et al.* (2007), ***Clinical Laboratory Standards Institute (CLSI) (2007).

The extracts were then combined, filtered through a Büchner funnell and concentrated by evaporation at 30˚C (Rotavapor® 210 R-210 BÜCHI) (Labortechnik AG, Flawil, 211 Switzerland). The extract obtained in each case was suspended in 100 ml of water. The resulting aqueous extracts were then lyophilized and the powder stored at −20˚C.

2.4. Antibacterial Activity of the Grape Extract

A first screening was performed to analyze the antibacterial activity of the different grape extracts (Tempranillo, Garnacha and Cabernet Sauvignon) against *C.jejuni* LP1. The extract which showed the strongest antibacterial activity was then selected for the following experiments with antibiotic-resistant strains. In all cases we used the following quantitative procedure: 1 ml of sample was transferred into different flasks containing 4 ml of BB. Bacterial inocula (50 µl with 1x108 colony forming units x mililiter (CFU/ml) were then inoculated into the flasks under aseptic conditions. All cultures were prepared in triplicate and incubated microaerobically at 42˚C for 24 h (130 rpm) in the VAIN. Positive growth controls were prepared by transferring 1 ml of saline solution (NaCl 0.9%) to 4 ml of BB and 50 µl of bacterial inocula. After incubation, serial decimal dilutions of mixtures were prepared in saline solution and they were plated (10 µl) onto fresh MHB agar and incubated microaerobically at 42˚C in the VAIN. The number of CFU was assessed after 48 h of incubation. Results were expressed as log CFU/ml. The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) were determined following the procedure described above and by using each grape extract diluted in sterile water to obtain the desired final concentration. MIC was defined as the lowest concentration of sample that provokes a statistically significant decrease in viability with respect to the control growth after 24 h of treatment. MBC was defined as the lowest concentration of sample where no growth was observed after 24 h of treatment.

2.5. Assessment of Total Phenolic Content (TPC)

The total phenolic content (TPC) in the different grape extracts was determined in accordance with the Folin-Ciocalteu micro method as previously described by Schmidt *et al.* [17]. Briefly, samples (10 µl) were added to a 96-well microtiter plate (Sarstedt, Nümbrecht, Germany) at an adequate dilution in triplicate. To start the reaction, 150 µl of aqueous Folin-Ciocalteu (Sigma-Aldrich) solution (14 ml water to 1 ml of Folin-Ciocalteu reagent) was added to each well. After 3 minutes, 50 µl of $NaHCO_3$ solution (2 ml of saturated $NaHCO_3$ to 3 ml of water) was added to each well and the plate was placed in the dark at room temperature for 2 h. Absorbance was measured at 725 nm using a BioTek Synergy HT Multi-Mode microplate reader (BioTek Instruments Inc., Vermont, USA), and the data were acquired and processed using BioTek's Gen5TM software (BioTek Instruments Inc.). Gallic acid (Sigma-Aldrich) was used as the standard for a calibration curve. TPC was expressed as milligrams of gallic acid equivalents per liter (mg GAE/L).

2.6. Determination of Individual Phenolic Compounds of the Extracts by HPLC and Mass Spectrometry Detection

All HPLC analyses were carried out on a Hewlett-Packard Agilent 1200 Series liquid chromatography system equipped with a quaternary pump and a photodiode array detector (DAD) (Agilent Technologies, Waldrom, Germany). The column used was a Phenomenex Luna C18 column (4.6 × 150 mm, 5 µm) (Phenomenex, California, USA) which was set thermostatically at 25˚C. Chromatographic data were acquired and processed using an Agilent Chemstation for LC 3D system (Rev. B.04.01) (Agilent Technologies). The HPLC method conditions were as described by Avila *et al.* [18]. Briefly, the binary mobile phase used for analyses were aqueous 4.5% formic acid (A) and HPLC-grade acetonitrile (B) at a flow rate of 0.5 ml/min. The elution started with 10% B, and the gradient was 20% B from 0 to 20 min, 25% B from 20 to 30 min, and 35% B from 30 to 50 min. Detection wavelengths were 280, 320, 440 and 520 nm and samples were analyzed in triplicate. Peaks were identified by comparing their retention time and UV-vis spectra with the reference compounds, and the data were quantified using the corresponding curves of the reference compounds as standards. In order to confirm the identity of

the recorded compounds, additional analyses were performed by using HPLC with mass spectrometry detection (HPLC-MS). For mass spectrometry an Agilent 1100 series liquid chromatograph/mass-selective detector equipped with a quadrupole (G1946D) mass spectrometer (Agilent Technologies) was used. Separation was achieved with an ORBAX Eclipse XDB-C18, (4.6 × 150 mm, 5 µm) (Agilent Technologies). Elution was performed with a gradient between 2.5% acetic acid in Milli-Q water (solution A), a mixture of 2.5% acetic acid in Milli-Q and water-acetonitrile (90:10) (solution B), and pure acetonitrile (solution C) at a flow rate of 0.5 ml/min, and an injection volume of 20 µl. The elution programme consisting of the following: from 100% A to 100% B in 3 min, from 100% to 93% B in 5 min, from 7% to 10% C in 7 min, from 10% to 15% C in 5 min, from 15% to 50% C in 5 min and isocratic 50% C and B for another 5 min. Electrospray ionisation in the positive mode was used. The electrospray capillary voltage was set to 2500 V, with a nebulising gas flow rate of 12 liters/min and a drying gas temperature of 150˚C.

2.7. Antimicrobial Activity Assay of Pure Phenolic Compounds

The phenolic compounds identified in the most active extract were tested against *C. jejuni* LP1 as pure compounds. The assayed compounds (quercetin, quercetin 3-glucoside, homovanillic acid, vanillic acid, gallic acid, protocatechuic acid, clorogenic acid, p-hidroxibenzoic acid, sinapic acid, catechin, epicatechin, and procyanidins B1, B2 were purchased from Sigma-Aldrich). The procedure used has been described above. CFU was assessed after 48 h of incubation. Results were expressed as log CFU/ml.

2.8. Statistical Analysis

Analysis of variance (ANOVA) was performed by SPSS 19.0 for Windows, version 19.0.0 (Dec. 2011).

3. Results and Discussion

3.1. Antibiotic Susceptibility Test for *Campylobacter* spp.

The results of the antibiotic susceptibility test are shown in **Table 3**. The sensitivity to antibiotics was dependent on the *Campylobacter* strain. Strains from international culture collections (11168, 11351, 7572 and 7571) were the most sensitive, presenting from null to 2 antibiotic resistances. On the other hand, recent isolates from clinical (LP2) and veterinary origin (CN1 and CNL4) were resistant to five antibiotics. Among antibiotics, the high percentage of resistances were found in ciprofloxacin (66.6%) and nalidixic acid (58.3%), both of them of the fluoroquinolones group, whereas all strains tested were sensitive to aminoglucosides (streptomycin and gentamicin), chloramphenicol and amoxicillin/clavulanic acid. It is known that bacteria often lose virulence by growth *in vitro*, and that the genetics basis for virulence may be expressed completely only during growth in vivo. This fact has been observed for *Campylobacter*s, where the comparation of the transcriptional profile of the original clonal isolate of *C. jejuni* 11168 and the genome-sequenced clone of *C. jejuni* 11168 showed important differences in gene expression [19]. Also, the use of repeatedly subcultured strains of *Helicobacter pylori* (*H. pylori*), a close-related microorganism, in virulence experiments, has shown the loss of several virulence properties respect to the original strain [20]. These results demonstrate the effect of laboratory culture and storage on virulence properties, suggesting the importance to use strains with low subcultures in studies of virulence and/or sensitivity to drugs. The behavior against fluoroquinolones has confirmed the striking increase in resistance of *Campylobacter* against these drugs in the last years, rendering now in a limited use of them in the treatment of campilobacteriosis in many regions [2]. Only *C. coli* (LP2) was resistant to erythromycin, one of the first choices in the antibiotic treatment of campilobacteriosis due to its low resistance rates (0% to 12%), although it is generally higher in *C. coli*, ranging from 0% to 50% [21]. Even if the majority of *C. jejuni* and *C. coli* are resistant to *β*-lactam agents, amoxicillin plus clavulanic acid has already been reported as effective [22], in accordance with the results obtained in the present work. Other drugs such as tetracycline and chloramphenicol can be alternative antibiotics, but up to 60% of strains may be resistant to tetracycline [23].

In the last years, it has been found that some compounds derived from plants can be active against antibiotic-resistant pathogens associated with foods, possibly by using different mechanisms of action [24]. For this reason, in the present work we evaluate the effect of three different grape extracts against *Campylobacter* in order to clarify its possible use as an antimicrobial.

3.2. Antibacterial Effect of Grape Extracts on *Campylobacter* spp.

The results of the antibacterial activity of the different grape extracts against *C. jejuni* LP1 showed that phenolic extraction using methanol as solvent (average 4.56 log of growth inhibition) was more effective than the extraction with water (average 1.91 log of growth inhibition) (**Table 4**). This is consistent with the total phenolic content (TPC) determined for each extract, which showed that the amount of phenolic compounds extracted using methanol was higher than the one obtained with water

Table 3. Antimicrobial susceptibility profile for the *Campylobacter* spp. strains by Kirby-Bauer disc diffusion method.

Antibiotic	LP1	1168	118	11351	CIII	CN1	CNL1	CNL2	7571	LP2	CNL4	7572
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	S
Cephalotin	S	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**
Streptomycin	S	S	S	S	S	S	S	S	S	S	S	S
Tetracyclin	I	S	S	S	I	**R**	**R**	**R**	S	I	**R**	S
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	S
Eritromycin	S	S	S	S	S	S	S	S	S	**R**	S	S
Aztreonam	S	S	S	**R**	**R**	**R**	S	S	S	**R**	**R**	**R**
Nalidixic Acid	**R**	S	**R**	S	S	**R**	**R**	**R**	S	**R**	**R**	S
Ciprofloxacin	**R**	S	**R**	S	**R**	**R**	**R**	**R**	S	**R**	**R**	S
Amoxicillin-Clavulanic Acid	S	S	S	S	S	S	S	S	S	S	S	S

S: susceptible; R: resistant; I: intermediate.

Table 4. Antibacterial activity of three different grape extracts against *C. jejuni* LP1. Three varieties of grapes (Cabernet Sauvignon, Garnacha and Tempranillo) were tested, and two different extractions were obtained (methanolic or water) for each grape variety. The results are expressed in Log CFU/ml ± standard deviation (SD) (n = 3).

Strain	Grape extract	TPC (mg GAE/L)	Median log CFU/ml ± SD (log reduction) (n = 3)		
			Control	Extract	Log Inhibition
C. jejuni LP1	Cabernet S. (M)	224.67	8.49 ± 0.1	4.78 ± 1.1a	3.71
	Cabernet S. (W)	103.53	8.2 ± 0.1	5.14 ± 0.8a	3.06
	Garnacha (M)	218.43	8.7 ± 0.1	3.92 ± 0.9a	4.78
	Garnacha (W)	103.53	8.24 ± 0.2	8.28 ± 0.6	0.00
	Tempranillo (M)	224.09	8.5 ± 0.1	3.32 ± 0.7a	5.18
	Tempranillo (W)	126.31	8.2 ± 0.1	6.67 ± 1.0a	2.13
	Cabernet S. (M)	224.67	8.49 ± 0.1	4.78 ± 1.1a	3.71

coinciding with the reported by others [25]. However, no differences were observed in the individual phenolic compounds extracted, which were the same independently of the extraction method used (data not shown). Among the methanolic extracts, the most effective was the Tempranillo extract. Thus, we selected it for next series of experiments.

The results of the antibacterial activity of the selected grape extract against different *Campylobacter* strains are presented in **Table 5**. For this assay, the five most resistant strains (with 4 or 5 antibiotic resistances) were used and the clinical isolate LP1 (with 2 antibiotic resistances) was also included. With the purpose to determine the lower concentration of the grape extract able to inhibit *Campylobacter* growth we calculated the MIC and MBC for each strain. The results obtained showed a relationship between strain and sensitivity. In all cases, the MIC was lower than 300 mg GAE/L, being the lowest value of 60 mg GAE/L for *C. jejuni* LP1 (two antibiotic resistances) and C. coli LP2 (five antibiotic resistances). The MIC for the other strains was 120 mg GAE/L (*C. jejuni* CN1, CNL1, and CNL2) and 180 mg GAE/L for *C. coli* CNL4. The MBC was between 240 mg GAE/L and 120 mg GAE/L, showing a similar behavior as MIC. In general terms the extract showed a strong capacity to inhibit *Campylobacter* growth regardless of the *Campylobacter* species (*C. jejuni* or *C. coli*) or the origin of the strain (human or veterinary). These MIC and MBC values seem relevant, taking into account that they are below 100 mg/L [26].

3.3. Phenolic Composition of the Grape Extract

In **Table 6** are shown the main individual phenolic compounds identified in the Tempranillo grape extract. The main group of phenolic compounds in the extract consisted of catechins (85.7%) and phenolic acids (13.7%).

Table 5. MIC and MBC of the Tempranillo grape extract against different antibiotic resistant strains of *Campylobacter* spp. The results are expressed in Log UFC/ml ± standard deviation (SD) (n = 2).

	Median log CFU/ml ± SD (log reduction) (n = 2)					
Concentration of the grape phenolic extract (mg GAE/L)	***Campylobacter* strain**					
	***C. jejuni* LP1**	***C. jejuni* CNL1**	***C. jejuni* CN1**	***C. jejuni* CNL2**	***C. coli* LP2**	***C. coli* CNL4**
Control	7.89 ± 0.2	8.04 ± 0.2	8.45 ± 0.2	8.39 ± 0.1	7.54 ± 0.2	8.01 ± 0.2
300	>1.48a* ± 0.0	>1.48a ± 0.0	>1.48a ± 0.0	>1.48a ± 0.0	>1.48a ± 0.0	>1.48a ± 0.0
240	>1.48a ± 0.0	>1.48a ± 0.0	>1.48a ± 0.0	>1.48a ± 0.0***	>1.48a ± 0.0	>1.48a ± 0.0**
180	>1.48a ± 0.0	>1.48a ± 0.0***	>1.48a ± 0.0***	4.90a ± 1.4	>1.48a ± 0.0	4.60a ± 0.2**
120	>1.48a ± 0.0***	5.38a ± 0.0**	6.45a ± 0.7**	6.75a ± 0.1**	>1.48a ± 0.0***	7.70 ± 0.1
60	2.77a ± 1.2**	8.40 ± 0.8	8.40 ± 0.1	8.49 ± 0.7	7.13a ± 1.0**	7.95 ± 0.2
30	8.58 ± 0.2	8.77 ± 0.2	8.70 ± 0.0	8.29 ± 1.1	8.65 ± 0.2	8.17 ± 0.0

*Calculated log of detection limit (30 CFU per plate). **Minimal Inhibitory Concentration (MIC), ***Minimal Bactericidal Concentration (MBC), a significantly different with respect to the growth control ($p \leq 0.05$).

Table 6. Individual phenolic composition (mg/L) of the Tempranillo grape extract (mean ± standard deviation) (n = 3) determined by HPLC-MS.

Family	Compound	Concentration (mg/L)
Flavonols	Quercetin-3-glucoside	4 ± 0.0
	Quercetin	7 ± 0.0
Phenolic Acids	Gallic Acid	37 ± 0.1
	Homogentisic Acid	21 ± 0.0
	Protocatechuic Acid	27 ± 7.0
	Chlorogenic Acid	6 ± 0.1
	Homovanillic Acid	72 ± 4.6
	Vanillic Acid	41 ± 2.0
	p-cumaric Acid	12 ± 0.0
	p-Hidroxibenzoic Acid	21 ± 1.6
	Sinapic Acid	31 ± 1.0
Catechins	Catechin (cat)	680 ± 4.4
	Epicatechin (ec)	806 ± 8.1
	B1 (cat-ec)	66 ± 1.3
	B2 (ec-ec)	130 ± 23.1
TOTAL		300* ± 0.0

*Total phenolic content (mg GAE/L).

Epicatechin and catechin were the major identified compounds, while homovanillic, vanillic and gallic acid were the most abundant within phenolic acids. The total phenolic compounds in the grape extract were quantified in 300 mg GAE/L. Vanillic and gallic acid moieties have been associated before with a loss of cytoplasmic membrane integrity, with the resultant loss of ion gradients, pH homeostasis and inhibition of respiratory activity [27]. On the other hand, the mechanisms of action proposed for the antibacterial activity of catechins have been mainly attributed to cytoplasmic membrane damage, although other mechanisms could be involved [28].

With the purpose to evaluate the impact of the phenolic compounds presented in the extract in the observed behavior, the pure phenolic compounds identified as part of the most active extract (homovanillic acid, vanillic acid, gallic acid, protocatechuic acid, clorogenic acid, p-hidroxibenzoic acid, catechin, epicatechin, and procyanidin dimmers B1 and B2) were tested against *C. jejuni* LP1 and results are shown in **Table 7**. Phenolics acids were the most active of the assayed compounds, provoking a growth decrease between 6.7 and 7.6 log, while epicatechin was the only flavanol with activity as pure compound (1 log). These results show the contribution of phenolic acids to the inhibition of *Campylobacter* growth, although as part of the extract, additive and/or synergistic effects could be involved in the behavior observed in the case of the grape extract. This fact was previously described by us for the GSE extract [12] and others are observed a similar behavior for some catechins [28] and for phenolic acids such as gallic acid [29].

4. Conclusion

In summary, the results obtained in this work indicate that grape extracts could be an important source of phenolic compounds potentially active against *Campylobacter*. The grape extract assayed has been demonstrated to be useful against *Campylobacter* strains with multiple antibiotic resistances. Phenolic acids have been identified as

Table 7. Effect of the standard phenolic compounds on the growth of *C jejuni* LP1. All the compounds were tested at 1 mg/ml, except B1 and B2, which activity was assayed at 0.5 mg/ml. The results are expressed in Log UFC/mL ± standard deviation (SD) (n = 2).

Median log CFU/ml ± SD (log reduction) (n = 2)				
Family	**Compound**	**Control**	**Compound Activity**	**Log of Inhibition**
Flavonols	Quercetin-3 -glucoside	8.28 ± 0.0	8.28 ± 0.0	NI**
	Quercetin	8.28 ± 0.0	8.28 ± 0.0	NI
Phenolic Acids	Homovanillic Acid	8.36 ± 0.1	>1.48a* ± 0.0	6.88
	Vanillic Acid	9.10 ± 0.1	>1.48a ± 0.0	7.62
	Gallic Acid	8.15 ± 0.0	>1.48a ± 0.0	6.70
	Protocatechuic Acid	8.78 ± 0.0	8.77a ± 0.1	NI
	Chlorogenic Acid	8.20 ± 0.1	8.17a ± 0.1	NI
	p-hidroxibenzoic Acid	8.15 ± 0.1	>1.48a ± 0.0	6.67
	Sinapic Acid	8.40 ± 0.0	8.42 ± 0.2	NI
Catechins	Catechin (cat)	8.31 ± 0.1	8.84 ± 0.1	NI
	Epicatechin (ec)	8.31 ± 0.1	7.27a ± 0.0	1.04
	B1 (cat-ec)	8.21 ± 0.1	8.61 ± 0.1	NI
	B2 (ec-ec)	8.21 ± 0.1	8.20 ± 0.1	NI

*Calculated log of detection limit (30 CFU per plate). **NI: no growth inhibition. a Significantly different with respect to the growth control ($p \leq 0.05$).

the main compounds related with the behavior observed, and they are usually the main phenolics in grape and grape-derived products [30], constituting an important metabolite derived from the metabolism of more complex phenolic compounds, such as anthocyanins [31]. This fact could contribute to standardizing the production process of grape extracts to inhibit *Campylobacter* growth.

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