Selective antibacterial effect on *Campylobacter* of a winemaking waste extract (WWE) as a source of active phenolic compounds

Elisa Mingo, Jose M. Silvan, Adolfo J. Martinez-Rodriguez *

Institute of Food Science Research (CIAL), CSIC-UAM, Department of Biotechnology and Microbiology - C/ Nicolás Cabrera, 9. Cantoblanco Campus, Autonoma University of Madrid, 28049, Madrid, Spain

*Corresponding author:

Tel: +34 91 001 79 64

Fax: +34 91 001 79 05

E-mail address: adolfo.martinez@csic.es

Abstract

The feasibility of winemaking waste (WW) as source of active phenolic compounds against *Campylobacter* was evaluated using a mixture of ethanol-water (50% v/v) as extraction solvent. The winemaking waste extract (WWE) was active against all the strains tested, and most of them were inhibited at WWE concentrations between 40-100 mg GAE/L. However, different lactic acid bacteria (LAB) strains were practically unaffected by WWE, suggesting a selective antibacterial effect of WWE against *Campylobacter*. The fractioning and phenolic characterization of WWE using HPLC-MS showed that catechins and proanthocyanidins were the main compounds involved in the antibacterial effect. Among them, the structural verification using pure phenolic standards showed that epicatechin gallate and resveratrol were the most active compounds against *Campylobacter*. We conclude that the study of the main mechanisms involved in the antibacterial activity against *Campylobacter* of epicatechin gallate, resveratrol, and other related compounds presenting gallate side-chain, can contribute for future design of useful antibacterials against this pathogen.

Keywords: winemaking waste, phenolic compounds, antibacterial activity, *Campylobacter*, epicatechin gallate

Introduction

Grapes used for the wine industry constitute around eighty percent of the worldwide grape production, being Spain the main producer (Fontana, Antonioli & Bottini, 2013). The largest fraction of winery waste is the winemaking waste (WW), consisting of the skins, seeds, and stems left after juice or wine is pressed. Red wine, fermented on the skins and then pressed, is the main source of WW. It is a complex mixture of polysaccharides, fermentation by-products, dietary fiber, and polyphenols amongst others (Yu & Ahmedna, 2013). Grapes are particularly rich in bioactive polyphenols, especially flavonoids, stilbenes, and phenolic acids. However, even though some polyphenols are extracted into wine, the majority remains in the WW, due to the poor extraction during the winemaking process (Friedman, 2014). In this regard, it is estimated that in Europe alone, 14.5 million tons of grape by-products are produced annually (Chouchouli, Kalogeropoulos, Konteles, Karvela, Makris & Karathanos, 2013). The large amount of waste produced after winemaking, in addition to being a great loss of valuable materials, also raises serious management problems, both from the economic and environmental point of view (Mateo & Maicas, 2015). For this reason, winery by-products arising from industry generates the need to find alternative ways for their utilization. The main industrial applications for WW include its use as source of various products such as ethanol, alcoholic beverages, tartaric and citric acid, fertilizer and dietary fiber (Teixeira, Baenas, Dominguez-Perles, Barros, Rosa, Moreno & Garcia-Viguera, 2014). However, some properties of WW are incompatible with agricultural requirements and therefore WW must be conditioned before use. For example, the high level of polyphenols in WW generate some concern when it is used as animal feed, due to its low digestibility (Baumgärtel, Kluth, Epperlein & Rodehutschord, 2007) or as fertilizer, causing inhibition in the germination of crops

(Negro, Tommasi & Miceli, 2003). However, this disadvantage may be turned to an advantage when WW is used as a source of polyphenols enriched extracts, thus combining corporate profitability with a significant advance in environmental protection around the winemaking zones (Anastasiadi, Chorianopoulos, Nychas & Haroutounian, 2009). There is extensive information available on the biological activities and potential health benefits of WW phenolic compounds (Teixeira et al., 2014; Friedman, 2014). Among their properties, the antibacterial character has gained interest in the last years due to their promising use as an alternative to traditional antibiotic and disinfectants (Friedman, 2014; Taylor, 2013). Between the foodborne bacterial pathogens affecting humans, campylobacters are the leading cause of bacterial diarrheal disease worldwide (Ganan, Silvan, Carrascosa & Martinez-Rodriguez, 2012). In the European Union, campylobacteriosis was the most commonly reported zoonosis, with 214,268 confirmed human cases in 2012 (EFSA Journal, 2014).

In the last years, the rise in the incidence of infections caused by antibiotic-resistant strains of *Campylobacter* makes this illness increasingly difficult to treat (Zhang & Plummer, 2008), and the same applies to the emergence of *Campylobacter* strains resistant to industrial disinfectants (Mavri, Kurinčič & Možina, 2012). Accordingly, the tendency for searching natural and sustainable strategies to reduce the incidence of *Campylobacter* is rising (Ganan et al., 2012). Some wine and grape phenolic compounds have previously proved to be effective against some *Campylobacter* strains (Silvan, Mingo, Hidalgo, de Pascual-Teresa, Carrascosa & Martinez-Rodriguez, 2013; Ganan, Martinez-Rodriguez & Carrascosa, 2009). For this reason, the main purpose of the present work was to evaluate the feasibility of WW as a source of active phenolic compounds against *Campylobacter*, also assessing its impact

on probiotic lactic acid bacteria (LAB) and establishing a relation between phenolic structure and antibacterial function.

2. Materials and methods

2.1. Extract preparation

The winemaking waste extract (WWE) was obtained from a WW of Tempranillo variety, following the procedure described by Pallauf, Rivas-Gonzalo, del Castillo, Cano & de Pascual-Teresa (2008) with some modifications. Briefly, WW was homogenized using an Ultra-Turrax T25 (IKA-Werke GmbH & Co., Staufen, Germany) for 5 min. to obtain 100 g of homogenate. 100 mL of ethanol-water (50% v/v) was added to the homogenate and mixed for 15 min. Afterwards, it was centrifuged for 10 min. at 4500 x g and the supernatant was collected. The extraction process was repeated twice more. The extracts were combined, filtered through a Büchner funnel and concentrated at 30°C using a rotary evaporator (BÜCHI Labortechnik GmbH, Essen, Germany). The extract obtained was made up to 100 mL of MilliQ® water. The aqueous extract was stored at -20°C.

2.2. Bacterial strains, growth media and culture conditions

The microorganisms used in this study included 12 different strains of *Campylobacter* (8 of *C. jejuni* and 4 of *C. coli*) and 10 strains of LAB (3 *Lactobacillus casei*, 2 *L. plantarum*, and 1 *L. salivarius*, *L. paracasei*, *L. rhamnosus*, *Enterococcus faecium*, and *Pediococcus damnosus*). Strain specification and origin of the specimen is provided in Table 1.

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 Mueller-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% N₂, 10% CO₂, 5% O₂) 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 (Elmi Ltd., Riga, Latvia) at 42°C for 24 h in microaerophilic conditions in the VAIN. These bacterial inocula cultures (~1 x 10⁸ colony forming units (CFU/mL) were used for the antibacterial activity assays.

For LAB, the liquid growth medium consisted of MRS broth (Laboratorios Conda, Madrid, Spain), supplemented with 10% agar for the plating medium. Cultures of LAB were prepared as follows: the frozen strain were reactivated by plating in MRS agar and incubated at 37°C in a 5% CO₂ humidified atmosphere (Inco 2, Memmert, Schwabach, Germany) for 48 h. Isolated colonies were then inoculated in MRS broth and incubated at 37°C at 130 rpm in an Infors HT Multitron humidified shaker (Infors HT, Bottmingen, Switzerland) for 24 hours. These bacterial inocula cultures (~1 x 10⁸ CFU/mL) were used for the assays.

2.3. Evaluation of the antibacterial activity of WWE

The antibacterial activity of WWE against *Campylobacter* and LAB strains was determined as follows: 1 mL of WWE was transferred into different flasks containing 4 mL of BB (for *Campylobacter* assays) or MRS (for LAB assays). Bacterial inocula (50 µL with ~1 x 10⁸ 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 (for *Campylobacter*) or at 37°C for 24 h (130 rpm) in the Infors incubator (for LAB). Positive growth controls were prepared using 0.9% saline solutions instead WWE. Minimal inhibitory concentration (MIC) was defined as the lowest amount of extract that provokes a significant quantitative decrease ($p < 0.05$) in the viability respect to the control growth after 24 h of treatment. Minimal bactericidal concentration (MBC) was defined as the lowest bactericidal concentration (detection limit 30 CFU per plate) of extract after 24 h of treatment. After incubation, serial decimal dilutions of mixtures were prepared in 0.9% saline solution and plated (20 μ L) onto fresh MHB agar and incubated microaerobically at 42°C in the VAIN. The CFU was assessed after 48 h of incubation. Results were expressed as log CFU/mL.

2.4. Characterization of the phenolic composition of WWE

The total phenolic content (TPC) in WWE was determined in accordance with the Folin-Ciocalteu micromethod as previously described by Silvan et al., (2013). Results were expressed as milligram of gallic acid equivalents per liter of extract (mg GAE/L). Individual phenolic compounds were determined by HPLC analyses and mass spectrometry detection (MS). 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 x 150 mm, 5 mm) (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 Silvan et al (2013). 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 was starting with 10% B, 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-MS. For MS analysis an Agilent 1100 series liquid chromatograph/mass-selective detector equipped with a quadrupole (G1946D) mass spectrometer (Agilent Technologies) was used, employing the same conditions described above for separation and elution. Electrospray ionization in the positive mode was used. The electrospray capillary voltage was set to 2500 V, with a nebulizing gas flow rate of 12 L/min and a drying gas temperature of 150°C.

2.5. Fractioning of WWE

The main phenolic compounds present in the WWE were fractioned by semi-preparative reverse-phase high-performance liquid chromatography (RP-HPLC) as previously reported Silvan et al. (2013). The fractions (F1, F2, F3 and F4) were collected automatically with a fraction collector model II (Waters Corp., Milford, Massachusetts, USA). The chromatographic run was repeated 20 times to obtain adequate sample volume to be used for the further analyses. The collected fractions were rotary evaporated at 35°C to reduce the volume. After that, the concentrates obtained for each fraction were completed to the total volume injected (5.5 mL) with distilled water. Samples were filter sterilized (0.22 µm, Sarstedt, Nümbrecht, Germany), and stored at -20°C until use. The collected fractions were used to determine the antibacterial activity against *C. jejuni* 11168. Phenolic composition of collected fractions was determined following the HPLC analyses and MS detection described above.

2.6. Verification of antibacterial response using pure standards

The main phenolic compounds identified in F2 (active fraction) were tested against *C. jejuni* 11168 viability as pure compounds. The assayed compounds (quercetin 3-glucoside, syringic acid, gallic acid, catechin, epicatechin, epicatechin-gallate, delphinidin-3-glucoside, t-resveratrol and t-piceid) were purchased from Extrasynthese (Lyon, France). Antibacterial resistance was determined using the broth microdilution method. This was carried out in BB and prepared in triplicate with an inocula of $\sim 1 \times 10^6$ CFU/mL using 96 well-microtitre plates (Sarstedt). Serial dilutions of each phenolic standard (5 to 1000 mg/L) were prepared. The wells without the addition of the phenolic standards were used as experimental controls. After 48 h of incubation in the VAIN at 42°C, the CFU was assessed as described above. Antibacterial activity was carried out in triplicate.

2.7. Statistical analysis

The results were reported as means $\pm$ standard deviation (SD). The data was analyzed by analysis of variance (ANOVA) with a post hoc Duncan test ($p < 0.05$). All statistical tests were performed with IBM SPSS Statistics for Windows, Version 19.0 (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. Antibacterial activity of the WWE against *Campylobacter*

The results of the antibacterial activity of WWE against *Campylobacter* strains are presented in Table 2. In a similar way as observed in previous works carried out using grape extracts or grape seed extracts (Silvan et al., 2013; Mingo, Carrascosa, de Pascual-Teresa & Martinez-Rodriguez, 2014), WWE was active against all the strains

studied. The grade of efficiency of the extract was related with the strain analyzed, being the MIC between 40-160 mg GAE/L and the MBC between 100-320 mg GAE/L. Most of the studied strains were inhibited at WWE concentrations between 40-100 mg GAE/L. These results demonstrate the efficacy of WWE as inhibitor of *Campylobacter* growth, in spite of the modifications in the phenolic fraction during winemaking (Friedman, 2014).

In practical terms, the antibacterial activity of a phenolic extract is very interesting in the case that it is observed at concentrations below 100 mg/L (Rios & Recio, 2005), because is not apparently very difficult to get this concentration in most of the extraction procedures to prepare natural extracts. In this work, the solvent used for the extract preparation was a mixture of ethanol-water (50% v/v). Several researchers have described that the presence of water in the extraction solvent used for grape by-products extraction increases the permeability of cell tissues, improving the recovery of water soluble compounds (Fontana et al, 2013). Although higher yields have been obtained using methanol as unique solvent (Fontana et al., 2013; Mingo et al., 2014) these results indicate that the use of an economic and environmentally-friendly solvent as ethanol can be enough to obtain an active WWE against *Campylobacter*. This is consequent with the fact that *Campylobacter* have proved to be more sensitive than others microorganisms to several phenolic compounds (Friedman, 2014).

3.2. Effect of WWE on probiotic lactic acid bacteria (LAB)

Different LAB species are integrated as part of human gut microbiota, positively contributing to the health and wellbeing. In the present work, we have evaluated the WWE effect on 10 potentially probiotic LAB strains. In Table 3 it is shown that the inhibitory effect of WWE on LAB strains was significantly lower than the observed for

Campylobacter strains. The highest concentration of the WWE used for *Campylobacter* studies (320 mg GAE/L, bactericidal for all the *Campylobacter* strains), only inhibited 1.5 log of LAB growth respect to experimental controls, while two strains (*L. casei* Ha and *L. casei* CIAL2) were not affected by WWE. This behavior suggests that WWE could have a selective effect on *Campylobacter* growth. Previously, other researchers have observed that LAB can be more resistant than other bacteria to phenolic compounds. For example, Lee, Jenner, Lowa & Lee (2006) found that strains of probiotic LAB and bifidobacteria were less affected by tea polyphenols than other bacterial groups. In the same way, Hervet-Hernandez, Pintado, Rotger & Goñi. (2009) observed that the polyphenols present in an extract of grape pomace did not inhibit the growth of *L. acidophilus* CECT 903, having a stimulatory effect on the growth of this strain. In the present work, we have observed a similar behavior for *L. salivarius* IFa strain. For this strain, the growth increased proportionally with increasing concentration of WWE (Table 3). Although there are significant differences between the LAB strains, the global behavior indicates that LAB growth was relatively unaffected by WWE.

3.3. Phenolic characterization of WWE

In Table 4 it is shown the composition in individual phenolic compounds of the WWE. Flavanols (catechins and proanthocyanidins) were the major components, accounting for around the 75.4% of the phenolic compounds in the extract. They were followed by flavonols (32.4%). Anthocyanins, phenolic acids and stilbenes were in minor proportion. Despite the effects of the fermentation and the mildest process used for phenolic extraction, the distributions in families of individual phenolic compounds were similar to those obtained from the GSE (Silvan et al., 2013) or grapes (Mingo et al., 2014). Also, in the WWE, t-resveratrol and t-piceid were found and quantified. The recovery of these compounds may be improved when using by-products of fermentation

due to the influence of variables such as yeast strain, maceration time, use of enzymes, aging, etc., during winemaking (Feijoo, Moreno & Falque, 2008). Within the catechins and proanthocyanidins, the most abundant compound was epicatechin (156.7 mg/L), followed by proanthocyanidin B2 (110.2 mg/L). Among the flavonols, the most abundant were quercetin-3-glucoside (94.4 mg/L) and quercetin-3-rhamnoside (89.4 mg/L). Malvidin-3-glucoside was the major anthocyanin, while the phenolic acids gallic and syringic acid were identified and quantified in similar proportions. These phenolic compounds have also been identified in others WWE (Jara-Palacios, Hernanz, Cifuentes-Gomez, Escudero-Gilete, Heredia & Spencer, 2015; Melo, Massarioli, Denny, dos Santos, Franchin, Pereira, Vieira, Rosalen & de Alencar, 2015; Yu & Ahmedna, 2013). The concentration of these compounds tends to vary, among others, due to variables such as grape variety and extraction process. Further, WWE was fractionated by RP-HPLC obtaining four fractions. The phenolic composition of these fractions is showed in Table 5. Fraction 1 (F1) included only gallic acid and proanthocyanidin B1, while fraction 2 (F2) was mainly formed by catechins and proanthocyanidins. In fraction 3 (F3) eluted the flavonols, anthocyanins, and t-resveratrol, while no phenolic compounds were found in fraction 4 (F4). The analysis of the antibacterial activity of each fraction against the most resistant strain tested (*C. jejuni* 11168) showed that the inhibitory effect observed in the whole WWE is concentrated in the compounds eluting in F2 (Figure 1). This result links the catechins and proanthocyanidins with the antibacterial effect of WWE. In addition to being the major phenolic compounds constituting the extract, there are several reports describing the antibacterial effect of catechins and proanthocyanidins against different bacteria (Nakayama, Shimatani, Ozawa, Shigemune, Tsugukuni, Tomiyama, et al., 2013; Reygaert, 2014). Moreover, we have previously observed some antibacterial effect of

pure epicatechin on *C. jejuni* (Ganan et al., 2009; Mingo et al., 2014). The fractioning of a commercial grape seed extract active against *Campylobacter* also suggested that some catechins were involved in the antibacterial effect of the whole extract (Silvan et al, 2013), however it was difficult to associate the effect to a specific compound or family of compounds, because three of the four obtained fractions had antibacterial activity against *Campylobacter*. Now, having only an active fraction (F2), we checked the individual response of each identified compound against *C. jejuni* using pure phenolic standards.

3.4. Verification of antibacterial effect using pure phenolic standards

The antibacterial activity of the selected phenolic standard against *C. jejuni* is shown in Table 6. All the pure phenolic compounds assayed were identified in the WWE and in the active fraction F2. The results showed that epicatechin gallate (ECG) was the most active compound, with a MIC of 10 mg/L and a MBC of 20 mg/L. In both cases, these values are lower than the concentration of this compound in WWE (25.9 mg/L; Table 4), suggesting its outstanding role in the antibacterial activity. ECG is one of the main catechins in grapes and green tea, and it has shown antibacterial activity against both gram-negative and gram positive bacteria, also acting synergistically with other phenolic compounds and antibiotics (Reygaert, 2014). This fact could be relevant taking in account that this compound is part of an extract enriched in phenolic compounds. The gallate side chain of this compound has been associated with its antibacterial activity (Friedman, Henika, Levin, Mandrell & Kozukue, 2006) and the main mechanisms of action described results from the compound binding to the bacterial lipid bilayer cell membrane, causing damage or dysfunction of the membrane (Reygaert, 2014; Cushnie & Lamb, 2011). Specifically in *Campylobacter*, the close related compound epigallocatechin gallate (EGCG) modifies *Campylobacter* multidrug

efflux systems, restoring macrolide efficacy in resistant strains (Kurinčič, Klančnik & Možina., 2012). Also as part of a grape seed extract, epicatechin gallate was in the most active fraction of phenolic compounds against *Campylobacter* (Silvan et al, 2013). Epicatechin, with a very low activity (MIC 1000 mg/L) and catechin (without antibacterial activity) were the other catechins analyzed. No pure standard was available for the dimer EC-ECG, but its similar structure, carrying a gallate side chain, suggest that it can be also involved in the observed behavior.

Another compound with a high antibacterial activity against *C. jejuni* was t-resveratrol, with a MIC of 20 mg/L and MBC of 50 mg/L. However, its concentration in the extract was much lower (7.2 mg/L), which is consistent with the fact that the fraction with higher content in t-resveratrol (F3) did not show antibacterial activity against *Campylobacter*. Nevertheless, the high concentration of glycosylated resveratrol (t-piceid) in the extract indicates that it can be a potential source of active resveratrol against *Campylobacter*.

The flavonol quercetin-3-glucuronide and the anthocyanin delphinidin-3-glucoside were no active against *C. jejuni*. Gallic and syringic acid had a similar behavior, with a MIC of 600 and 400 mg/L, respectively. These values are higher than the concentrations of these compounds in the extract (between 20 and 26 mg/L) showing that apparently they are not involved directly in the antibacterial effect of WWE.

4. Conclusions

The results obtained in this work evidences that WW can be a good source to obtain active phenolic extracts against *Campylobacter*. Phenolic compounds remaining in fermentation by-products are active against *Campylobacter* and can be extracted

using mild procedures, probably due to the effect of enzymatic activity and other previous winemaking procedures. Phenolic extracts active against *Campylobacter* have a smooth effect on probiotic LAB, suggesting a selective character of the inhibitory effect. Structure-function relationships were established for WWE, indicating that epicatechin gallate was the most active phenolic compound against *Campylobacter*. Moreover, WWE was rich in t-piceid, a potential source of resveratrol, also with high antibacterial activity against *Campylobacter*. Future work should be directed to know the mechanisms involved in the antibacterial activity against *Campylobacter* of epicatechin gallate, resveratrol, and other related compounds presenting gallate side-chain, contributing in this way to design useful antibacterial against this pathogen. Antibacterials based in phenolic compounds are potentially applicable in the entire target sites of food chain where campylobacters remain, and could be a cheaper and feasible tool to fight against this pathogen.

Acknowledgements

This work was funded through Projects AGL 2009-07894 and AGL 2013-47694-R from the CSIC, and CSD2007-00063FUN-C-FOOD (CONSOLIDER INGENIO 2010).

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Figure 1. Antibacterial activity of the WWE collected fractions against *C. jejuni* 11168. Results are the mean $\pm$ standard deviation of three determinations. Bars marked with the same letter indicate absence of significant differences by Duncan test ($p < 0.05$).

Table 1. Source of *Campylobacter* and Lactic Acid Bacteria (LAB) strains obtained from clinical, veterinary, chicken food chain and collection libraries.

Bacteria	Strain	Origin	Source
<i>C. jejuni</i>	118	Clinical	Hospital Carlos III, Madrid
	CIII	Clinical	Hospital Carlos III, Madrid
	LP1	Clinical	Hospital La Paz, Madrid
	CN1	Veterinary	CIAL ^a
	11168	Collection	NCTC ^b
	11351	Collection	NCTC
	CIAL1	Chicken food chain	CIAL
	CIAL2	Chicken food chain	CIAL
<i>C. coli</i>	LP2	Clinical	Hospital La Paz, Madrid
	CIAL3	Chicken food chain	CIAL
	CIAL4	Chicken food chain	CIAL
	CIAL5	Clinical	CIAL
<i>L. casei</i>	Ha	Food	CIAL
	CIAL1	Food	CIAL
	CIAL2	Food	CIAL
<i>L. plantarum</i>	748	Reference	CECT ^c
	749	Reference	CECT
<i>L. salivarius</i>	IFa	Food	CIAL
<i>L. paracasei</i>	LC01(Hansen)	Food	CIAL
<i>L. rhamnosus</i>	53103	Reference	ATCC ^d
<i>E. faecium</i>	BIFI58	Food	CIAL
<i>P. damnosus</i>	20331	Reference	DSMZ ^e

^a Collection from Institute of Food Science Research (CIAL).

^b Bacterial cultures obtained from the National Collection of Types Cultures (NCTC), UK.

^c Bacterial cultures obtained from the Colección Española de Cultivos Tipo (CECT), Spain.

^d Bacterial cultures obtained from the American Type Culture Collection (ATCC), USA.

^e Bacterial cultures obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Germany.

Table 2. Antibacterial activity of WWE against *Campylobacter* strains.

Strains	WWE concentration (mg GAE/L)					
	0	40	100	160	240	320
<i>C. jejuni</i> LP1	8.3 ^a ± 0.1	8.3 ^a ± 0.0	2.2 ^b ± 0.1	>1.5 ^{*c}	>1.5 ^{*c}	>1.5 ^{*c}
<i>C. jejuni</i> CIII	8.1 ^a ± 0.1	8.1 ^a ± 0.2	6.2 ^b ± 0.1	>1.5 ^{*c}	>1.5 ^{*c}	>1.5 ^{*c}
<i>C. jejuni</i> CN1	8.4 ^a ± 0.1	8.8 ^a ± 0.0	4.5 ^b ± 0.3	>1.5 ^{*c}	>1.5 ^{*c}	>1.5 ^{*c}
<i>C. jejuni</i> 118	8.6 ^a ± 0.1	5.0 ^b ± 0.2	>1.5 ^{*c}	>1.5 ^{*c}	>1.5 ^{*c}	>1.5 ^c
<i>C. jejuni</i> 11168	8.6 ^a ± 0.3	8.8 ^a ± 0.2	8.7 ^a ± 0.1	6.0 ^b ± 0.1	4.3 ^c ± 0.3	>1.5 ^{*d}
<i>C. jejuni</i> 11351	8.1 ^a ± 0.1	8.4 ^a ± 0.5	7.1 ^b ± 0.1	3.5 ^c ± 0.3	>1.5 ^{*d}	>1.5 ^{*d}
<i>C. jejuni</i> CIAL1	8.3 ^a ± 0.1	7.7 ^b ± 0.3	6.9 ^c ± 0.1	2.2 ^d ± 0.3	>1.5 ^{*e}	>1.5 ^{*e}
<i>C. jejuni</i> CIAL2	9.0 ^a ± 0.2	8.8 ^a ± 0.2	7.1 ^b ± 0.2	4.1 ^c ± 0.1	1.8 ^d ± 0.2	>1.5 ^{*e}
<i>C. coli</i> LP2	8.5 ^a ± 0.1	8.4 ^a ± 0.1	6.1 ^b ± 0.2	3.1 ^c ± 0.1	>1.5 ^{*d}	>1.5 ^{*d}
<i>C. coli</i> CIAL3	9.0 ^a ± 0.1	8.6 ^b ± 0.2	7.2 ^c ± 0.2	6.1 ^d ± 0.2	2.2 ^e ± 0.2	>1.5 ^{*f}
<i>C. coli</i> CIAL4	8.1 ^a ± 0.2	8.1 ^a ± 0.2	7.1 ^b ± 0.2	5.0 ^c ± 0.2	2.9 ^d ± 0.2	>1.5 ^{*e}
<i>C. coli</i> CIAL5	9.0 ^a ± 0.1	8.9 ^a ± 0.1	7.9 ^b ± 0.2	5.2 ^c ± 0.3	2.7 ^d ± 0.1	>1.5 ^{*e}

Results are the mean ± standard deviation of three determinations. Values in the same row marked with the same superscript letter indicate absence of significant differences ($p < 0.05$).

* Calculated log of detection limit (30 CFU per plate).

Table 3. Antibacterial activity of WWE against lactic acid bacteria strains (LAB).

Strains	Concentration WWE (mg GAE/L)					
	0	40	100	160	240	320
<i>E. faecium</i> BIFI58	8.2 ^a ± 0.1	8.2 ^a ± 0.3	8.3 ^a ± 0.1	8.2 ^a ± 0.1	8.3 ^a ± 0.1	7.9 ^b ± 0.1
<i>L. casei</i> CIAL1	8.2 ^a ± 0.2	8.3 ^a ± 0.2	8.4 ^a ± 0.2	8.4 ^a ± 0.3	8.0 ^b ± 0.2	8.0 ^b ± 0.1
<i>L. casei</i> CIAL2	7.7 ^a ± 0.2	7.8 ^a ± 0.2	7.7 ^a ± 0.2	7.8 ^a ± 0.2	7.6 ^a ± 0.3	7.5 ^a ± 0.2
<i>L. casei</i> Ha	7.4 ^a ± 0.1	7.4 ^a ± 0.4	7.6 ^a ± 0.2	7.7 ^a ± 0.3	7.5 ^a ± 0.1	7.4 ^a ± 0.2
<i>L. paracasei</i> LC01	7.5 ^a ± 0.1	7.5 ^a ± 0.1	7.3 ^a ± 0.2	7.4 ^a ± 0.1	6.5 ^b ± 0.2	6.0 ^c ± 0.1
<i>L. plantarum</i> 748	8.8 ^a ± 0.1	8.7 ^a ± 0.2	8.7 ^a ± 0.3	8.4 ^b ± 0.2	8.3 ^b ± 0.2	8.0 ^c ± 0.1
<i>L. plantarum</i> 749	9.2 ^a ± 0.1	9.1 ^a ± 0.2	9.3 ^a ± 0.2	9.1 ^a ± 0.2	9.2 ^a ± 0.1	8.5 ^b ± 0.2
<i>L. rhamnosus</i> 53103	9.0 ^a ± 0.1	9.2 ^a ± 0.1	8.9 ^a ± 0.1	8.9 ^a ± 0.2	8.6 ^b ± 0.2	8.4 ^b ± 0.2
<i>L. salivarius</i> IFa	8.2 ^b ± 0.1	8.3 ^b ± 0.1	8.4 ^b ± 0.2	8.6 ^a ± 0.1	8.6 ^a ± 0.1	8.7 ^a ± 0.1
<i>P. damnosus</i> 20331	8.2 ^a ± 0.2	8.0 ^b ± 0.3	7.8 ^b ± 0.2	7.3 ^c ± 0.2	7.1 ^c ± 0.2	7.1 ^c ± 0.3

Results are the mean ± standard deviation of three determinations. Values in the same row marked with the same superscript letter indicate absence of significant differences by Duncan test ($p < 0.05$).

Table 4. Individual phenolic composition of WWE (mg/L of extract).

Phenolic compound	mg/L
Flavonols	
Myricetin-3-glucoside	47.6 ± 0.4
Quercetin-3-rutinoside	24.0 ± 0.3
Quercetin-3-glucoside	94.4 ± 0.7
Quercetin-3-rhamnoside	89.4 ± 0.5
Quercetin-3-glucuronide	70.3 ± 0.6
Kaempferol-3-rutinoside	10.1 ± 0.1
Kaempferol-3-glucoside	28.0 ± 0.5
Quercetin	37.9 ± 1.1
Kaempferol	15.2 ± 2.1
Phenolic acids	
Syringic acid	20.4 ± 0.4
Gallic acid	26.0 ± 1.3
Flavanols	
Catechin (CAT)	88.0 ± 6.6
Epicatechin (EC)	156.7 ± 12.2
B1 (EC-CAT)	83.3 ± 4.3
B2 (EC-EC)	110.2 ± 9.0
Procyanidin trimer	96.1 ± 10.4
Epicatechin gallate (ECG)	25.9 ± 1.5
Procyanidin dimmer gallate	18.1 ± 0.2
Anthocyanins	
Delphinidin-3-glucoside	23.1 ± 0.3
Peonidin-3-glucoside	71.3 ± 0.6
Malvidin-3-glucoside	125.7 ± 0.8
Malvidin-3-glucoside-acetate	1.8 ± 0.1
Stilbenes	
t-Resveratrol	7.2 ± 0.0
t-Piceid	27.9 ± 0.7
TPC^a	2060.2 ± 104.3

Results are the mean ± standard deviation of three determinations.

^a Total phenolic content is expressed as milligram of gallic acid equivalents per liter (mg GAE/L extract).

Table 5. Phenolic composition of the WEE collected fractions (mg/L of extract) obtained by RP-HPLC.

Compound	F1	F2	F3	F4
Flavonols				
Myricetin-3-glucoside			6.2 ± 0.1	
Quercetin-3-rutinoside	n.d.	n.d.	18.4 ± 0.0	n.d.
Quercetin-3-glucoside	n.d.	n.d.	63.4 ± 0.2	n.d.
Quercetin-3-rhamnoside	n.d.	n.d.	7.0 ± 0.0	n.d.
Quercetin-3-glucuronide	n.d.	21.6 ± 0.0	38.1 ± 0.1	n.d.
Kaempferol-3-rutinoside	n.d.	n.d.	5.4 ± 0.1	n.d.
Kaempferol-3-glucoside	n.d.	n.d.	16.0 ± 0.2	n.d.
Quercetin	n.d.	n.d.	15.1 ± 0.1	n.d.
Kampferol	n.d.	n.d.	5.3 ± 0.2	n.d.
Phenolic acids				
Syringic acid	n.d.	12.7 ± 0.5	n.d.	n.d.
Gallic acid	23.9 ± 1.6	0.7 ± 0.0	n.d.	n.d.
Flavanols				
Catechin (CAT)	n.d.	9.8 ± 0.5	n.d.	n.d.
Epicatechin (EC)	n.d.	23.5 ± 0.8	n.d.	n.d.
B1 (EC-CAT)	34.2 ± 3.9	17.5 ± 0.5	n.d.	n.d.
B2 (EC-EC)	n.d.	41.1 ± 0.2	n.d.	n.d.
Procyanidin trimer	n.d.	16.2 ± 0.5	n.d.	n.d.
Epicatechin gallate (ECG)	n.d.	11.2 ± 0.4	n.d.	n.d.
Procyanidin dimmer gallate	n.d.	13.6 ± 0.1	n.d.	n.d.
Anthocyanins				
Delfinidin-3-glucoside	n.d.	12.7 ± 0.6	n.d.	n.d.
Peonidin-3-glucoside	n.d.	0.7 ± 0.0	36.9 ± 0.5	n.d.
Malvidin-3-glucoside	n.d.	1.1 ± 0.1	85.6 ± 0.3	n.d.
Malvidin-3-glucoside-acetate	n.d.	n.d.	0.3 ± 0.0	n.d.
Stilbenes				
t-Resveratrol	n.d.	1.0 ± 0.0	3.9 ± 0.0	n.d.
t-Piceid	n.d.	1.8 ± 0.0	17.3 ± 0.2	n.d.

Results are the mean ± standard deviation of three determinations.

n.d. = non detected.

Table 6. Antibacterial activity of pure standards of phenolic compounds identified in WWE collected fraction F2. Fraction was obtained from WWE by RP-HPLC.

Concentration (mg/L)	Pure standards of phenolic compounds identified in fraction F2							
	Q3G	SA	GA	CAT	EC	ECG	D3G	RV
0	8.8 ^a ± 0.2	9.8 ^a ± 0.6	9.4 ^a ± 0.8	9.2 ^a ± 0.4	9.5 ^a ± 0.4	8.9 ^a ± 0.3	8.6 ^a ± 0.2	9.2 ^a ± 0.4
5	8.6 ^a ± 0.4	9.6 ^a ± 0.4	9.2 ^a ± 0.4	9.4 ^a ± 0.3	9.7 ^a ± 0.2	8.8 ^a ± 0.5	8.4 ^a ± 0.4	8.9 ^a ± 0.3
10	8.7 ^a ± 0.2	9.5 ^a ± 0.3	9.4 ^a ± 0.6	9.2 ^a ± 0.5	9.3 ^a ± 0.5	3.6 ^b ± 0.2	8.6 ^a ± 0.3	9.1 ^a ± 0.5
20	8.7 ^a ± 0.4	9.8 ^a ± 0.3	9.2 ^a ± 0.6	9.6 ^a ± 0.4	9.5 ^a ± 0.3	> 1.5 ^{*c}	8.3 ^a ± 0.6	6.3 ^b ± 0.2
50	8.5 ^a ± 0.4	9.4 ^a ± 0.6	9.5 ^a ± 0.4	9.4 ^a ± 0.3	9.4 ^a ± 0.2	> 1.5 ^{*c}	8.4 ^a ± 0.2	> 1.5 ^{*c}
80	8.8 ^a ± 0.3	9.3 ^a ± 0.6	9.4 ^a ± 0.6	9.2 ^a ± 0.2	9.6 ^a ± 0.3	> 1.5 ^{*c}	8.5 ^a ± 0.4	> 1.5 ^{*c}
100	8.7 ^a ± 0.4	9.7 ^a ± 0.4	9.3 ^a ± 0.3	9.5 ^a ± 0.2	9.6 ^a ± 0.5	> 1.5 ^{*c}	8.6 ^a ± 0.3	> 1.5 ^{*c}
200	8.8 ^a ± 0.3	9.7 ^a ± 0.6	9.5 ^a ± 0.4	9.3 ^a ± 0.4	9.5 ^a ± 0.3	> 1.5 ^{*c}	8.6 ^a ± 0.2	> 1.5 ^{*c}
400	8.8 ^a ± 0.3	7.1 ^b ± 0.6	9.3 ^a ± 0.2	9.3 ^a ± 0.3	9.6 ^a ± 0.2	> 1.5 ^{*c}	8.4 ^a ± 0.2	> 1.5 ^{*c}
600	8.6 ^a ± 0.4	6.4 ^c ± 0.7	6.2 ^b ± 0.2	9.2 ^a ± 0.5	9.5 ^a ± 0.4	> 1.5 ^{*c}	8.3 ^a ± 0.5	> 1.5 ^{*c}
800	8.4 ^a ± 0.4	4.1 ^d ± 0.2	5.0 ^c ± 0.6	9.5 ^a ± 0.5	9.6 ^a ± 0.2	> 1.5 ^{*c}	8.4 ^a ± 0.3	> 1.5 ^{*c}
1000	8.7 ^a ± 0.4	3.0 ^e ± 0.4	4.8 ^c ± 0.7	9.3 ^a ± 0.6	8.2 ^b ± 0.3	> 1.5 ^{*c}	8.4 ^a ± 0.2	> 1.5 ^{*c}

Results are the mean ± standard deviation of three determinations. Values in the same column marked with the same superscript letter indicate absence of significant differences by Duncan test ($p < 0.05$). Q3G (Quercetin-3-glucoside), SA (Syringic acid), GA (Gallic acid), CAT (Catechin), EC (Epicatechin), ECG (Epicatechin gallate), D3G (Delphinidin-3-glucoside), RV (Resveratrol).

* Calculated log of detection limit (30 CFU per plate).