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Antibacterial activity of a grape seed extract and its fractions against *Campylobacter* spp.

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ABSTRACT

In this study, the antibacterial activity of a grape seed extract (GSE) was examined against different *Campylobacter* strains. Growth inhibition was in the range from 5.08 to 6.97 log CFU/ml, demonstrating the strong capacity of the GSE to inhibit *Campylobacter* growth. Further dilution of the extract showed a minimal inhibitory concentration (MIC) of 20 mg/l and a minimal bactericidal concentration (MBC) of 60 mg/l against *Campylobacter jejuni*. GSE was fractioned by RP-HPLC and phenolic composition was determined by HPLC-DAD and HPLC-MS. The phenolic profile of GSE mainly consisted on flavonols, phenolic acids, catechins and proanthocyanidins, and anthocyanins. Among them, catechins and proanthocyanidins were the major compounds, representing 77.6% of total phenolic compounds determined. The analysis of the antibacterial activity against *C. jejuni* of the collected fractions showed that phenolic acids, catechins and proanthocyanidins were the main responsible of the behavior observed. These results showed that identification and quantification of the individual phenolic compounds of GSE could be feasible to standardize the production process to obtain an enriched extract potentially useful to control *Campylobacter* in the food chain.

1. Introduction

Campylobacter species are the leading cause of bacterial foodborne diarrheal illness worldwide (Ganan, Silván, Carrascosa, & Martinez-Rodriguez, 2012). The *Campylobacter* genus comprises 17 species, 14 of which have been associated with human illnesses, and of these, *Campylobacter jejuni* and *Campylobacter coli* causes more than 95% of the infections attributed to this genus (Park, 2002). Clinical spectrum of enteric disease caused by *Campylobacter* ranges from severe inflammatory to moderate non-inflammatory diarrhea. The severity of symptoms mainly depends on the infective strain and on the physical condition of the host (Blaser & Engberg, 2008). In a small percentage of cases, long term and potentially serious complications, such as Guillain–Barré syndrome, Reiter's syndrome or reactive arthritis can arise (Lastovica, 2006). Although most cases of campylobacteriosis are self-limiting, antimicrobial treatment is usually required in patients with severe or prolonged enteritis, especially in infants or the elderly, in immunocompromised individuals, and in cases of extra-intestinal manifestations. Increase in the incidence of infections caused by antibiotic-resistant strains of *Campylobacter* makes this illness increasingly difficult to treat (Zhang & Plummer, 2008). Many sources and vehicles of *Campylobacter* infection in humans have been suggested (Acke et al., 2006; Lee & Newell, 2006; Stanley & Jones, 2003), although the most common is mainly associated with the consumption and/or handling of poultry meat, especially fresh broiler meat (Humphrey, O'Brien, & Madsen, 2007). A large proportion of the European Union (EU) chicken production is contaminated with *Campylobacter* (EFSA, 2010) and new concerns have arisen due to the recent EU ban on the use of antibiotics in animal feed to promote growth (European Parliament and of the Council, 2003). For all these reasons, the development of new potential antibacterial compounds for controlling *Campylobacter* is necessary, with the main purpose to find alternative solutions to the use of antibiotics for controlling this pathogen. Consumer concerns about the safety of food has 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. Among them, grape seed extracts (GSE) have shown a broad range of bioactive properties, including antimicrobial

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activity (Ahn, Grün, & Mustapha, 2007; Brown, Huang, Haley-Zitlin, & Jiang, 2009; Chedea, Braicu, Chirila, Ober, & Socaciu, 2011; Rhodes, Mitchell, Wilson, & Melton, 2006). Also, GSE has recently been marketed as a dietary supplement due to its beneficial effects and free radical-scavenging ability (Mandic, Dilas, Cetkovic, Canadanovic-Brunet, & Tumbas, 2008), and it has been considered effective as additive in retarding lipid oxidation to different foods related with *Campylobacter* contamination, such as raw chicken and beef products (Kulkarni, De Santos, Kattamuri, Rossi, & Brewer, 2011; Selani et al., 2011). However, there are no previous reports in the literature about the effect of GSE on *Campylobacter* and the phenolic compounds involved in it, in spite of its importance as a foodborne pathogen. In the present work, we report the investigation on the antibacterial activity of a GSE against different species and strains of *Campylobacter*, identifying the compounds responsible of the behavior observed with the purpose to establish the relationship between structure and antibacterial function, also discussing its potential use to control *Campylobacter* contamination in foods.

2. Materials and methods

2.1. Extract preparation

Grape seed powder (Laboratorios GSN, Madrid, Spain) was purchased from a local market in Spain. The powdered grape seeds (500 mg) was dissolved in 10 ml of distilled water and stirred for 5 min at room temperature. The suspension obtained was centrifuged at 4000 rpm for 10 min and the supernatant was collected and sterilized by filtration with a pore size membrane of 0.22 μm (Sarstedt, Nümbrecht, Germany). The GSE obtained was stored at −20 °C until use.

2.2. Bacterial strains, growth media, and culture conditions

Twelve *Campylobacter* strains were tested for the antibacterial activity: *C. jejuni* LP1 and *C. coli* LP2 (strains of clinical origin provided by Hospital La Paz, Madrid), *C. jejuni* CIII and *C. jejuni* 118 (strains of clinical origin provided by Hospital Carlos III, Madrid), *C. jejuni* CN1, *C. jejuni* CNL1, *C. jejuni* CNL2 and *C. coli* CNL4 (strains isolated from chickens provided by a poultry producer), *C. jejuni* NCTC 11351 and *C. jejuni* NCTC 11168 were bought from the National Collection of Type Cultures (NCTC, London, UK), *C. jejuni* ATCC 33291 and *C. coli* ATCC 43478 were bought from the American Type Culture Collection (ATCC, Virginia, USA). 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 ($\sim 1 \times 10^8$ CFU/ml) were used for the antibacterial activity assays.

2.3. Antibacterial activity

The antibacterial activity of GSE and its collected fractions was analyzed using two procedures: a quantitative method and a qualitative method. The quantitative procedure was as follows: 1 ml of samples (GSE or collected fractions) was transferred into different flasks containing 4 ml of BB. Bacterial inocula (50 μl with 1×10^8 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 sterile water to 4 ml of BB and 50 μl of bacterial inocula. After incubation, serial decimal dilutions of mixtures were prepared in saline solution (0.9% NaCl) and they were plated (20 μl) onto fresh MHB agar and incubated microaerobically at 42 °C in the VAIN. The number of colony forming units (CFU) was assessed after 48 h of incubation. Results were expressed as log CFU/ml. Determination of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) was determined following the procedure described above and by using GSE or collected fractions diluted in sterile water to obtain the desired final concentration. MIC was defined as the lowest amount of extract or fraction that provokes a significant quantitative decrease ($p < 0.05$) in viability respect to the control growth after 24 h of treatment. MBC was defined as the lowest bactericidal concentration (detection limit 30 CFU per plate) of extract or fraction after 24 h of treatment.

The qualitative method was used as screening method to check the effect of the different concentrations of GSE and collected fractions without using serial dilutions respect to the control growth (visual reduction of growth). In the procedure, after the treatment described above, 20 μl of the mixtures without dilution were platted onto MHB agar and incubated microaerobically at 42 °C for 48 h in the VAIN.

2.4. Assessment of total phenolic content (TPC)

The concentration of TPC in the GSE and its collected fractions was determined in accordance with the Folin-Ciocalteu micromethod as previously described by Schmidt, Erdman, and Lila (2005). Briefly, samples (10 μl) were added to a 96-well microtiter plate (Sarstedt) at an adequate dilution in triplicate. To start the reaction, 150 μl of aqueous Folin-Ciocalteu (Sigma–Aldrich, Missouri, USA) solution (14 ml water to 1 ml of Folin–Ciocalteu reagent) was added to each well. After 3 min, 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 Gen5™ 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).

2.5. Determination of individual phenolic compounds by HPLC analyses 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 C_{18} 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. (2009). 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