Olive mill wastewater as a potential source of antibacterial and anti-inflammatory compounds against the food-borne pathogen *Campylobacter*

Jose Manuel Silván^a, Manuel Alejandro Pinto-Bustillos^b, Pablo Vásquez-Ponce^a, Marin Prodanov^b, Adolfo J. Martinez-Rodriguez^{a,*}

^a *Grupo de Microbiología y Biocatálisis, Departamento de Biotecnología y Microbiología, Instituto de Investigación en Ciencias de la Alimentación (CIAL), CSIC-UAM - C/ Nicolás Cabrera, 9. Campus Cantoblanco, Universidad Autónoma de Madrid, 28049, Madrid, Spain*

^b *Grupo de Ingredientes Alimentarios Funcionales, Departamento de Producción y Caracterización de Nuevos Alimentos, Instituto de Investigación en Ciencias de la Alimentación (CIAL), CSIC-UAM - C/ Nicolás Cabrera 9. Campus Cantoblanco, Universidad Autónoma de Madrid, 28049, Madrid, Spain*

*Corresponding author at: Instituto de Investigación en Ciencias de la Alimentación (CIAL), CSIC-UAM, Departamento de Biotecnología y Microbiología. C/ Nicolás Cabrera, 9. Campus Cantoblanco, Universidad Autónoma de Madrid, 28049, Madrid, Spain.

E-mail address: adolfo.martinez@csic.es (A. J. Martinez-Rodriguez)

Abstract

Phenolics and secoiridoids from crude olive mill wastewater (OMW) were purified using a preparative hybrid separation process: pressure-driven cross-flow membrane micro-(MF) and ultrafiltration (UF) and solid phase extraction (SPE). Five fractions with different contents of these compounds were obtained and their antibacterial activity against several strains of *Campylobacter* was evaluated. The results showed that the fraction with the highest content of phenolic and secoiridoid compounds had a relevant antibacterial activity against all tested strains with a strain-dependent character. Pre-treatment of LPS-stimulated cells with this fraction decreased the production of nitric oxide (NO), showing its potential as anti-inflammatory agent. Finally, the effect of the same fraction on the viability of human intestinal epithelial cell lines was evaluated. In conclusion, OMW could be useful as a potential source of bioactive compounds against *Campylobacter*, increasing the valorisation of olive oil by-products.

Keywords: olive mill wastewater (OMW), cross-flow membrane filtration, solid phase extraction (SPE), *Campylobacter*, antibacterial activity, anti-inflammatory activity

1. Introduction

Food wastes are a great concern, since serious environmental issues are posed and limited options for their resolution are available. However, over the last years, these wastes have been the focus of a growing interest due to their content in potentially high added-value compounds (Zinnai et al., 2016). Good examples of such matter are the olive oil by-products, which retain the biggest part of phenolic and secoiridoid compounds (~98%) that are not transferred to olive oil (Venturi et al., 2017; Araújo et al., 2015). Olive oil production is widespread worldwide, but is especially substantive in the Mediterranean countries. Europe produces around 78% of the world's olive oil and Spain is the largest producer in the European Union with approximately 60% of the total European production (IOC, 2016). Olive oil production is characterized by the generation of substantial amounts of solid, liquid and gaseous residues, which management is a challenge issue for the olive mill operators from both, economic and environmental perspectives. The amount of these residues differs according to the technology applied for the extraction of olive oil. In the last decades three-phase and two-phase centrifugation processes have been applied by the majority of olive mills (Roig et al., 2006). The three-phase centrifugation system generates three fractions at the end of the process: a solid residue fraction called olive pomace, oil and a highly polluting aqueous waste, known as olive mill wastewater (OMW). On the other hand, the two-phase centrifugation system produces two fractions, oil and a heavy aqueous suspension of olive solids, which is used for olive pomace oil recovery and is the main pollutant in this production (Jerman Klen & Mozetič Vodopivec, 2012). These by-products require a complex management and disposal system due to their negative effects on the environment (Hentati et al., 2016; Justino et al., 2012). Several chemical, physical and biological methods have been applied for the treatment of OMW and olive pomace, but generally, these methods are costly and

not efficient to eliminate pollution problems (Chowdhury et al., 2013). However, OMW may have a great economic interest because they contain bioactive constituents such as Oleuropein (Cioffi et al., 2010), Hydroxytyrosol (Rubio-Senent, et al., 2012), Oleochantal, Oleacein and other minor phenolics and secoiridoid compounds (Venturi et al., 2017; Suárez et al., 2010), which have been associated with antioxidant, anti-inflammatory, and antimicrobial properties (Bulotta et al., 2013). Furthermore, these OMW compounds may act against specific foodborne pathogens, being a potential alternative to the use of antibiotics or chemical disinfectants. Among the foodborne pathogens, *Campylobacter* is the leading cause of bacterial gastroenteritis worldwide (Kaakoush et al., 2015). Infections by *Campylobacter* in humans are generally caused by consuming contaminated foods of animal origin, with poultry, especially chicken being the main reservoir, although *Campylobacter* has also been found in cattle, swine, sheep, wild birds and pets (Ganan et al., 2012). In the European Union, campylobacteriosis was the most commonly reported zoonosis, with 229213 confirmed human cases in 2015. In Spain, 13227 cases were reported, despite the fact that the coverage of the notification rates is estimated around 45% (EFSA Journal, 2016). In most cases, *Campylobacter* infection symptoms range from severe inflammatory to moderate non-inflammatory diarrhoea, but serious extraintestinal infection does rarely occur (Facciola et al., 2017). A hallmark of campylobacteriosis is the inflammation of the intestinal mucosa. Although this immune activity is essential for development of an appropriate response to infection, an exacerbate immune response could induce tissue damage. For this reason, it would be important to modulate the immune response avoiding a tissue damage which could trigger a chronic inflammatory bowel disease (Silvan et al., 2017). Infection with *Campylobacter* is also associated with other rare post-infectious complications including Guillain-Barré syndrome, reactive arthritis, or Miller Fisher Syndrome (O'Brien, 2017). Although most

cases of campylobacteriosis are self-limiting, antimicrobial treatment is usually required in patients with severe or prolonged enteritis, especially in infants or elderly people, and in immunocompromised individuals. In the last years, the rise in the incidence of infections caused by antibiotic-resistant strains of *Campylobacter* makes campylobacteriosis increasingly difficult to treat (ECDC, EFSA & EMA, 2017). In this context, the development of new potential antimicrobials for controlling *Campylobacter* is needed. Taking into account the vast potential offered by natural antimicrobial compounds, there is increasing research into the efficacy of constituents derived from plants, animals, bacteria and fungi, as well as organic acids, essential oils and bacteriocins (Hayek et al., 2013; Tiwari et al., 2009). In this regard, different food by-products, such as chitosan (Ganan et al., 2009; Mengibar et al., 2011; Olaimat et al., 2014), citrus by-products (Ganan et al., 2010; Castillo et al., 2017), fruit pomace (Salaheen et al., 2018) and winemaking by-products (Mingo et al., 2016; Silvan et al., 2013) have proven to have antibacterial activity against *Campylobacter*. However, studies about the antimicrobial effects of OMW on *Campylobacter* are scarce, in spite of the impact of *Campylobacter* as a foodborne pathogen.

In the present work, the antibacterial activity of several fractions obtained from OMW was evaluated against different *Campylobacter* strains. The anti-inflammatory properties and the phenolic and secoiridoid composition of the active fraction were also determined. Finally, the influence on cell viability of the antibacterial fraction was assessed in two human intestinal cell lines (Caco-2 and HT-29).

2. Material and methods

2.1 Materials and reagents

For HPLC analysis, milli-Q grade water was produced in-house by a Milli-Q® Integral 3 purification system (Merck Millipore, MA, USA). Methanol (HPLC grade) was purchased from Scharlab (Barcelona, Spain) and glacial acetic acid, from Sigma-Aldrich (Madrid, Spain). Demineralized water with electrical conductivity of 7-8 $\mu\text{S}/\text{cm}$ was obtained in-house by a reverse osmosis unit (Genius 300, Filtec Depuradoras, Girona, Spain). Hydroxytyrosol (3,4-dihydroxyphenyl ethanol o 3,4-DHPE) (> 98%), Tyrosol (4-hydroxyphenyl ethanol o 4-HPE) (98%), Quercetin-3-O-rutinoside (Rutin) and Oleuropein (3,4-DHPE-EA-Glu) (> 98%) reference substances were purchased from Sigma-Aldrich. 3,4-Dihydroxyphenylglycol (3,4-DHPG) (75%) was provided by Prof. Juan Fernández-Bolaños from the Instituto de la Grasa (CSIC) (Sevilla, Spain). For cell cultures, 3,4,5-Dimethylthiazol-2,5-diphenyl-tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were acquired from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin (5000 U/mL), phosphate buffered saline (PBS), and trypsin/EDTA solution (170,000 U/L) were purchased from Lonza (Barcelona, Spain). Fetal bovine serum (FBS) of South American origin (Hyclone, GE Healthcare, Logan, UK) was obtained from Thermo Fisher Scientific (Madrid, Spain). Cell culture dishes were obtained from Sarstedt (Barcelona, Spain).

2.2. Preparative fractionation of OMW, equipment and separation methodologies

OMW was provided by an olive mill company from the region of Toledo (central Spain) that uses 3 phase separation decanter system for olive oil production. It was obtained from well-ripened olives (cv. Cornicabra), collected in January 2017. OMW was collected directly from the decanter, frozen immediately and kept at -20°C until fractionation. Three separation techniques were used to obtain a clarified raw OMW (COMW) and five COMW derived fractions (F1-F5):

a) Pilot scale pressure-driven cross-flow membrane microfiltration unit. Around 20 L of raw OMW were first centrifuged in a Heraeus Multifuge RS3+ (Waltham, MA, USA) at 24,000 *g* for 20 min. The supernatants were combined and submitted to a fine clarification process by a multichannel mineral (0.5 μm pore size, 0.1 m^2 filtration surface) microfiltration (MF) membrane (Ceramem Co., MA, USA), coupled to a pilot scale pressure-driven cross-flow filtration unit as described by Morales et al (2018). Filtration was carried out in a continuous concentration mode, at constant transmembrane pressure of 0.8 bar at 20°C. At these conditions 18.2 L of OMW were concentrated to 7 L of dense suspension which was discarded. An amount of 11.5 L of clarified OMW was recovered. An aliquot of 250 mL of the clarified OMW (COMW) was freeze-dried and stored in dark at 4°C.

b) Preparative scale pressure-driven cross-flow membrane ultrafiltration (UF) unit. A macromolecular (colloidal) fraction was separated from COMW by a 10 kDa molecular mass cut-off (0.54 m^2 filtration surface, regenerated cellulose spiral-wound) membrane (model Prep-scale 6) from Millipore (Merck, Darmstadt, Germany). The membrane cartage was connected to a low pressure UF unit, consisting of a 5 L feed vessel, peristaltic pump with variable flow (1-13 L/min) and pressure gauge (1-5 bar). UF was carried out in a continuous concentration mode, at a constant transmembrane pressure of 0.8 bar and temperature of 20°C. An amount of 11.25 L of the COMW was concentrated to 1.5 L. Recovery of macromolecules was carried out by diafiltration of the 1.5 L of concentrate with addition of water (6 L). The purified macromolecular fraction (F1) was freeze-dried and stored in dark at 4°C. In the other hand, a total amount of 9.75 L of ultrafiltrate was obtained. An aliquot of 250 mL of this fraction (F2) was freeze-dried and stored in dark at 4°C.

c) Preparative scale solid phase extraction unit: The ultrafiltered OMW (F2) was further treated by a solid phase extraction (SPE) at preparative scale. An amount of 1 L of XAD7HP (macroreticular aliphatic acrylic cross linked polymer) resin from Rohm & Haas (Philadelphia, USA) was packed in a 2 L of glass tube, having a glass frit at the bottom (to retain the resin) and Teflon valve. An aliquot of 1.5 L was loaded to the resin at a flow rate of 60 mL/min and the permeate was recovered as F3. The resin was diafiltered with demineralised water until 0% of total soluble substances were measured at the exit of the column (by refractometry) and the collected diafiltrate was recovered as fraction F4. Both fractions were freeze-dried and stored in vacuum-sealed polyethylene bag in dark at 4°C. Phenolic and secoiridoid compounds were eluted with 96% ethanol (fraction F5). The solvent was recovered by distillation and the dry extract was redissolved with 50-100 mL of water, freeze-dried and stored in dark at 4°C. A summary of OMW clarification and its fractionation pathway is presented in **Figure 1**.

2.3. Bacterial strains, growth media and culture conditions

C. jejuni NCTC 11168, used as a reference strain, was bought from the National Collection of Type Cultures (NCTC) (London, UK). Seventeen *Campylobacter* strains were isolated from two different sources: chicken food chain (FC) (8 strains) and clinical patients (C) (9 strains). The isolation sources and species of the strains used in this study are shown in **Table 1**. All isolates were carried out in Burgos (Spain) between the years 2011-2014. The strains were stored at -80°C in Brucella Broth (BB) (Becton, Dickinson, & Co., NJ, USA) with 20% glycerol until use. The agar plating medium consisted of Mueller-Hinton agar supplemented with 5% defibrinated sheep blood (MHB) (Becton, Dickinson, & Co.). Liquid growth medium for *Campylobacter* strains consisted in BB. The frozen strains were propagated 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 40°C for 48 h. Isolated colonies were inoculated into 50 mL of BB and incubated under stirring at 150 rpm on an orbital shaker at 40°C for 24 h in microaerophilic conditions in the VAIN. These bacterial inoculum cultures ($\sim 1 \times 10^8$ CFU/mL) were used for the different experimental assays.

2.4. Antibacterial activity of COMW and COMW derived fractions

The antibacterial activity of COMW and its derived fractions against *C. jejuni* 11168 was evaluated following the procedure described by Silvan et al. (2013). Briefly, 1 mL of COMW and fractions F1 to F5 (2 mg/mL final concentrations) were transferred into different flasks containing 4 mL of BB. Bacterial inoculum (50 μ L of $\sim 1 \times 10^8$ CFU/mL) was then inoculated into the flasks under aseptic conditions. Culture was prepared in triplicate and incubated in the VAIN in the conditions described above. Positive growth controls were prepared by transferring 1 mL of sterile water to 4 mL of BB and 50 μ L of bacterial inoculum. After incubation, serial decimal dilutions of mixtures were prepared in saline solution (0.9% NaCl). Then, they were plated (20 μ L) onto fresh MHB agar and incubated in the VAIN. The colony forming unit (CFU) was assessed after 48 h of incubation. Results were expressed as log of CFU/mL.

After confirmation of the efficacy of the fractions against the reference strain, antimicrobial activity of the antibacterial fraction (F5) was determined for different *Campylobacter* strains (FC and C strains). For this purpose, a micro-method was employed and the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined as follows: sterile 96-well flat-bottom plates (Sarstedt) were used. BB (240 μ L) was dispensed into all wells of the microtiter plate. Each fraction was tested in several concentrations (0.25; 0.43; 0.62; 0.87; 1.0; 1.25; 1.50; 1.75; and 2 mg/mL), diluted in BB, and added to the wells (60 μ L). Bacterial inoculums

(3 μL of $\sim 1 \times 10^8$ CFU/mL) were added into appropriate well. Bacterial growth controls for each strain without fraction addition were also prepared. Plates were incubated in the VAIN in the same conditions described before. In order to determine the MIC and MBC of the tested fraction concentrations, 5 μL from each well were plated onto MHB and incubated for 48 h in the VAIN. MIC was defined as the lowest amount of fraction that provokes a decrease in viability respect to the control growth (visual reduction of growth) after 24 h of treatment (calculated in approximately 4 log of inhibition). MBC was defined as the lowest bactericidal concentration of fraction after 24 h of treatment.

2.5. Anti-inflammatory properties of COMW and COMW derived fractions with antibacterial activity

The mouse macrophage cell line RAW 264.7 was obtained from ATCC and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were plated at a density of 1×10^6 cells in 75 cm^2 culture flasks and maintained at 37°C under 5% CO_2 in a humidified incubator until 90% of confluence. Culture medium was changed every 2 days. After a confluent monolayer appeared, subculturing cell process was carried out.

Before the anti-inflammatory experiments, it was necessary to find out if the selected F5 fraction was toxic for RAW 264.7 macrophage cell line. For this, viability of macrophage cell line was determined by MTT assay as previously described (Silvan et al., 2017). Raw 264.7 cells were seeded in 96-well plates (5×10^4 cells/well) and allowed to adhere for 24 h. After that, the medium was removed and cells were washed with PBS. Then, cell medium was replaced with serum-free medium containing different concentrations of the antibacterial F5 fraction and cells were incubated at 37°C for 24 h. Control cells (non-treated) were incubated in serum-free medium. After that, the medium was replaced by 200 μL of serum-free medium and 20 μL of a MTT solution in PBS (5

mg/mL) were added to each well for the quantification of the living metabolically active cells after 1 h incubation. Formazan crystals in wells were solubilized in 200 μ L of DMSO. Absorbance was measured at 570 nm wavelength using a microplate reader Synergy HT (BioTek Instruments Inc., Winooski, VT, USA). Cell viability was calculated considering controls containing serum-free medium as 100% viable. Data represent the mean and standard deviation of three independent experiments ($n = 3$). All experiments were carried out between passage 10 to passage 30 to ensure cell uniformity and reproducibility.

For inflammatory experiments, 5×10^4 cells/well were seeded in a 96-well plate. Nitrite accumulation, and indicator of nitric oxide synthesis, was measured in the culture medium of treated and control cells by the Griess reaction. After 24 h of incubation, the medium was removed, cells were washed with 200 μ L PBS and treated with 0.01 mg/mL of the antibacterial fraction (F5) and 10 μ g/mL of LPS from *E. coli* O55:B5 for 24 h. Control cells were incubated in serum-free medium for 24 h. Finally, the media were collected and used for nitric oxide (NO) quantification. Briefly, 100 μ L of collected supernatant were plated in 96-well plate and an equal amount of Griess reagent constituted by 1% (w/v) sulfanilamide and 0.1% (w/v) N-1-(naphthyl) ethylenediamine-diHCl in 2.5% (v/v) H_3PO_4 , was added. The plate was incubated for 5 min and the absorbance was measured at 550 nm in a microplate reader Synergy HT. The amount of NO was calculated using a sodium nitrite standard curve (0-10 μ g/mL). Percent inhibition was calculated relative to control cells stimulated with 10 μ g/mL LPS.

2.6. HPLC-PAD-MS assessment of phenolic and secoiridoid compounds

Phenolic and secoiridoid compounds were analysed by a model LC 920 high performance liquid chromatograph (HPLC) from Varian (Palo Alto, CA, USA), using a photodiode array detector (PAD). The equipment was coupled to a quadrupole mass

spectrometer (MS) with electrospray ionisation source (ESI) (Hewlett-Packard, series 1100 MSD) (Palo Alto, CA, USA). Separation was carried out according to the methodology developed for assessment of highly polar dipoles from natural sources by Grases et al. (2015). ESI operation parameters were as described in Prodanov et al. (2013). Chromatograms were acquired at 240, 280 and 330 nm and mass spectra from m/z 100 to 2500 u . Quantification was carried out for compounds for which there were available reference substances: 3,4-Dihydroxyphenylglycol (3,4-DHPG), Hydroxytyrosol (3,4-DHPE), Tyrosol (4-HPE), Quercetin-3-O-rutinoside (Rutin), Luteolin-7-O-glucoside and Oleuropein. Hydroxytyrosol glucoside (3,4-DHPE-glu), Hydroxytyrosol hydroxydecarboxymethyl elenolate (3,4-DHPE-HDCM-EA) and Hydroxytyrosol decarboxymethyl elenolic acid (3,4-DHPE-DCM-EA, Oleacein) were quantified as Hydroxytyrosol equivalents, using the calibration curve of Hydroxytyrosol. Tyrosol glucoside (4-HPE-glu) was quantified in the same way, using the calibration curve of Tyrosol.

2.7. Effect of F5 COMW fraction with antibacterial activity on the viability of human intestinal epithelial cell lines

Cell viability was determined as described above in section 2.5. Human intestinal HT-29 and Caco-2 cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were plated at densities 1×10^5 cells in 75 cm^2 tissue culture flasks and maintained at 37°C under 5% CO_2 in a humidifier atmosphere. The culture medium was changed every two days. Confluent stock cultures were trypsinized (Trypsin/EDTA) and cells were seeded in 96-well plates ($\sim 5 \times 10^4$ cells per well) and incubated in culture medium at 37°C under 5% CO_2 in a humidifier incubator for 24 h. Briefly, cell medium was replaced with serum-free medium containing different concentrations of the active F5 fraction (0.25 to 2 mg/mL final concentrations) and the

cells were incubated at 37°C under 5% CO₂ for 24 h. Control cells were incubated in serum-free medium without fraction addition. Thereafter, 20 µL of a MTT solution in PBS (5 mg/mL) were added to each well for the quantification of the living metabolically active cells after 1 h. of incubation. Formazan crystals in wells were solubilized in 200 µL DMSO. Absorbance was measured at 570 nm wavelength using a microplate reader Synergy HT. Cell viability was calculated considering controls containing serum-free medium as 100% viable. Data represent the mean and standard deviation of three independent experiments (n = 3). All experiments were carried out between passage 10 to passage 30 to ensure cell uniformity and reproducibility.

2.8. Other physicochemical measurements

Total suspended solids were estimated indirectly by measuring turbidity with a TN100 infrared turbidimeter (Thermo-Fisher Scientific, Spain), attuned for measurements in the interval of 0 to 800 NTU (nephelometric turbidity units).

2.9. Statistical analysis

Results were reported as mean value ± standard deviations (SD) performed in triplicate. Data were subjected to statistical analysis by one-way analysis of variance (ANOVA) followed by The Tukey least significant differences (LSD) test for multiple comparisons. Differences were considered significant at $p < 0.05$. All statistical tests were performed with IBM SPSS Statistics for Windows, Version 21.0 (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. Preparative purification of phenolic and secoiridoid compounds from COMW by a hybrid MF/UF/SPE process

OMW is a very complex by-product of the olive oil production, containing a high load of suspended solids (3-15%) sugars (12%), pectins and other polysaccharides, polyols, proteins, phenolics, secoiridoids, minerals, and residual oil (Dermeche et al., 2013). Recovery of these molecules requires also a complex purification process. The first step of this purification is essential and involves complete separation of the abundant fine suspended solids in the crude OMW. The cross-flow membrane MF equipment, used in this work, reduced the turbidity of the centrifuged crude OMW from 362 to 0.47 NTU in the clarified OMW (COMW). COMW was obtained practically free of insoluble particles and oil.

The next UF treatment allowed the recovery of the colloidal COMW compounds as a singular fraction F1. The composition of this fraction will be evaluated in further studies, as it contains chromatographically (by means of HPLC) inseparable macromolecular complexes. Capasso et al. (2002) isolated a similar macromolecular fraction from OMW (polymerin) and showed that it was basically composed of polymeric carbohydrates, melanins and proteins. Further purification by preparative SPE allowed the complete separation of sugars, polyols, acids, and minerals (F3 and F4) from phenolic and secoiridoid compounds (F5) (data not shown). It is important to point out that the dry matter of both F3 and F4 fractions, represents around 75% of the dry matter of COMW, which means that a 4-fold enrichment in phenolic and secoiridoid compounds should be expected in the F5 fraction.

3.2. Antibacterial activity of COMW fractions

Results regarding the antibacterial properties of COMW and COMW derived fractions (2 mg/mL final concentration) against *C. jejuni* 11168 are shown in **Figure 2**. F5 was the only fraction with significant antibacterial activity ($p < 0.05$), being bactericidal after 24 h of treatment and reducing *Campylobacter* growth in more than 9 logs. COMW

and the other COMW fractions did not have significant antibacterial activity ($p>0.05$). These results demonstrate the strong capacity of the fraction F5 to inhibit *Campylobacter* growth. According to the described results, recent studies have also demonstrated the antibacterial effects of phenolic and secoiridoid enriched fractions obtained from OMW on several bacterial pathogens, including gram positive and gram negative bacteria (Aissa et al., 2017; Belaqziz et al., 2017). Usually, the antibacterial effect is greater in gram-positive than gram-negative bacteria, which is consistent with a thicker and more complex cell wall in the case of gram-negative bacteria (Leouifoudi et al., 2015; Obied et al., 2007). This behaviour is also consequent with one of the most cited mechanisms of action for phenolic extracts obtained from OMW: the disturbing of the cytoplasmic membrane (Serio et al., 2010; Casas-Sanchez et al., 2007). Although different bacteria have been assayed with the purpose of knowing their sensitivity to phenolic compounds from OMW, tests on *Campylobacter* are scarce and difficult to find in the literature, probably due to the specific requirements needed for working with this pathogen. However, some authors have recently observed that supplementing diet of broilers with different amounts of OMW extract and dehydrated olive cake results in a significant decrease in *Campylobacter* contamination (Branciari et al., 2016), suggesting that olive waste by-products could be useful to reduce the risk of *Campylobacter* diffusion in the chicken flock and consequently in meat. In order to evaluate the response of different *Campylobacter* species (*C. jejuni* and *C. coli*) isolated from different sources (chicken food chain and clinical) we assessed the MIC and MBC of several strains against the active F5 COMW fraction at different concentrations (from 0.25 to 2 mg/mL). The obtained results showed that as same as for the reference strain, F5 fraction had a relevant antibacterial activity against all the strains tested with a strain-dependent character (**Table 2**). The MBC of all strains was between 0.43 and 2 mg/mL, and more than 94 % of the

tested strains (16 of 17) had an MBC lower than 2 mg/mL. MIC for F5 fraction ranged from 0.25 to 1.75 mg/mL, and 65% of the strains had a MIC $\leq$ 1 mg/mL, which is considered very interesting for natural extracts from a practical point of view (Rios & Recio, 2005). Interestingly, when the average values obtained in each case for MIC and MBC were analysed, *C. coli* strains (food chain and clinical) were, in general, more resistant to bactericidal and inhibitory effect of F5 fraction than *C. jejuni* strains. This result is in agreement with the fact that these strains of *C. coli* also showed resistance to a greater number of antibiotics (unpublished data), which coincides with several results reported in recent years about the emergence of strains of *C. coli* with a high level of antibiotic resistance (EFSA and ECDC, 2017). Further studies using a greater number of strains will be conducted in order to assess the practical impact of the emergence of these resistant strains of *C. coli*. Likewise, it has been found in this study that clinical strains were more resistant to the antimicrobial effect of F5 fraction than those from the chicken meat food chain, suggesting that infectious strains may have some virulent attributes that make them more resistant to the effect of antimicrobial compounds. As mentioned above, this behavior should be studied in future works using a higher number of strains.

3.3. Anti-inflammatory properties of the F5 COMW fraction.

Inflammation associated with campylobacteriosis may progress to a chronic inflammatory process involving significant tissue damage (Louwen & Hays, 2013). Hence, there is growing interest in identifying compounds that contribute to modulate the severity of this inflammatory process. With this purpose, the anti-inflammatory activity of the COMW fraction with antimicrobial activity (F5) against *Campylobacter* was evaluated. During inflammation, inducible NO synthase (iNOS) is responsible for the elevated production of NO, which in turn can lead to tissue injury at the inflammatory site, organ dysfunction and inflammation-related disease (Conforti & Menichini, 2011).

Figure 3 shows the anti-inflammatory effect of F5 fraction on NO production in LPS-stimulated macrophages. The experiment was carried out using a lower concentration of the extracts than in those used in the antibacterial activity assay (0.01 mg/mL), as higher concentrations affected the viability of macrophages (data not shown). LPS significantly stimulated the production of NO in macrophages from 0.37 µg/mL (control value) to 3.99 µg/mL. Pre-treatment of LPS-stimulated cells with F5 fraction attenuated significantly ($p < 0.05$) the production of NO, provoking approximately a 66% of inhibition in the NO production. This fact demonstrates the potential anti-inflammatory activity of the F5 fraction. Previous studies have shown that OMW may present anti-inflammatory activity, mainly due to the presence of a number of phenolic compounds such as Hydroxytyrosol and Tyrosol, which can reduce the inflammatory responses to LPS by inhibiting the expression of iNOS (Sato et al., 2016; Aparicio-Soto et al., 2015; Zhang et al., 2009). In addition, some investigations have demonstrated that olive polyphenols can contribute to the modulation of the inflammatory responses by inhibition of various inflammatory mediators, cytokines and chemokines, such as IL6, IL8, TNF- α , and MCP-1 (Vezza et al., 2017; Ryu et al., 2015; Richard et al., 2011), which have been associated with the inflammatory response to campylobacteriosis (Hamza et al., 2017; Silvan et al., 2017).

3.4. Identification and distribution of phenolic and secoiridoid compounds in the COMW fractions.

The employed HPLC-PAD-MS methodology allowed the identification of 18 peaks (**Table 3**). Six of them, 3,4-Dihydroxyphenylglycol (3,4-DHPG), Hydroxytyrosol (3,4-DHPE), Tyrosol (4-HPE), Quercetin-3-O-rutinoside (Rutin), Luteolin-7-O-glucoside and Oleuropein (3,4-DHPE-EA-glu) were identified unambiguously by co-elution and comparison with mass and UV spectra and retention time of commercial reference substances. The rest of the peaks were identified tentatively, taking into account their

chromatographic (retention time and order of elution), spectroscopic (ultraviolet-visible (UV-VIS) spectra), and spectrometric (molecular and typical fragment ions) characteristics, as well as bibliography data (Bajouba et al., 2016; Michel et al. 2015; Jerman Klen, 2014). It should be mentioned that there was other peaks in the chromatogram with spectral characteristics typical for secoiridoids, but their identification and quantitative assessment was not possible because of the lack of reference substances.

As it can be seen from **Table 4**, the results show that elimination of macromolecules (F1) from COMW by UF did not lead to enrichment of the phenol content of the resulting F2 fraction. Rather, they indicate certain losses of most of the analysed compounds and suggest that the use of a membrane with molecular mass cut-off of 10 kDa was not entirely appropriate. A significant enrichment in phenolics and secoiridoids was observed only in the F5 fraction, which was obtained after sugar and mineral removal (F3 and F4) by SPE. In this case, enrichments of up to 520% (5.2-fold) were achieved for some molecules (Oleacein). However, it should be emphasized, that this enrichments were quite variable and specific for each molecule, in spite of that all of them proceeded from the same fraction (treatment). At this moment, we cannot offer reasonable explanation for these differences.

In general, the results (**Table 4**) suggest that the higher bioactivity of F5 fraction is directly related to the largest amount of phenolic and secoiridoid compounds present in F5. Phenolic compounds and their secoiridoid derivatives such as Hydroxytyrosol, Hydroxytyrosol and Tyrosol glucosides and Oleacein were among the main components of this fraction. Phenolics have been widely described as key antibacterial compounds in OMW, being Hydroxytyrosol and Oleuropein some of the main identified antimicrobial compounds (Thielmann et al., 2017; Leouifoudi et al., 2015; Yangui et al., 2010; Obied et al., 2007). Using pure reference substances of both compounds, we studied their

antibacterial effect on the *C. jejuni* 11168 strain at a final concentration of 1 mg/mL. Obtained results revealed that Hydroxytyrosol and Oleuropein did not show any antimicrobial activity against *Campylobacter*, and apparently, were not directly involved in the antibacterial effect observed for F5 fraction (data not shown). Other authors have also described a poor antibacterial activity for these compounds against *E. coli*, *H. pylori*, and other related bacteria, suggesting that their hydrolysis products would be more active as antibacterial compounds (Thielmann et al., 2017; Medina et al., 2009). Among hydrolysis products, these authors highlight the Oleacein, which is structurally similar to some commercial biocides, such as glutaraldehyde and *ortho*-phthalaldehyde (OPA) (Medina et al., 2013; Medina et al., 2009). In this work, at least one glutaraldehyde-like compound (Oleacein) has been found at significant amounts (92.68 mg/g) as a part of F5 fraction (**Table 4**), which suggests that this type of compounds may be involved in the observed inhibitory behavior. Other hydrolytical products identified in F5 fraction, such as Elenolic acid and some of its derivatives (3,4-DHPE-HDCM-EA), have also been proven to possess stronger bactericidal potential compared with Oleuropein (Thielmann et al., 2017, Bisignano et al., 2014; Mosele et al., 2014; De Marco et al., 2007; Soler-Rivas et al., 2000). In addition, it was shown that mixtures of phenols and secoiridoids exert greater antibacterial activity with respect to single compounds (Obied et al., 2007). Therefore, further work should be done to isolate and purify these compounds from OMW in order to confirm their antibacterial efficacy establishing a structure-function relationship.

3.5. Effect of F5 COMW fraction on the viability of human intestinal epithelial cell lines

In this study, it has been previously described how F5 COMW fraction, in a concentration range between 0.25 to 2 mg/mL, affected the viability of RAW 264.7, a murine leukemia macrophage cell line used as experimental model in the anti-

inflammatory studies. Then, the impact of this fraction on the viability of human intestinal cell lines HT-29 and Caco-2 was also studied. The obtained results are shown in **Figure 4**. F5 fraction affected the viability of human intestinal cells, reducing significantly ($p < 0.05$) the viability in both cell lines in the evaluated range of concentrations (from 0.25 to 2 mg/mL). The reduction of cell viability was more pronounced for HT-29 than Caco-2 cells, demonstrating the influence of cell type on the observed behavior. As these two cell lines are derived from human colon tumors, some authors describe this cell viability inhibition as an anti-proliferative activity (Bernini et al., 2015; Ramos et al., 2013). Consequently, this inhibitory effect could be associated with some of the phenolic compounds present in OMW, considering this behavior as a potential antitumor activity. In this line, Bassani et al (2016) have described the ability of an OMW extract rich in Hydroxytyrosol to inhibit the growth of colon cancer cells *in vitro*, also observing inhibition of tumor cell growth in 6-8 weeks old syngenic BalbC mice. These authors conclude that the reuse of OMW represents a promising strategy to both limit environment pollution and recovery of water-soluble phenols with potential chemopreventive properties for colon cancer treatment. Olive mill by-products have also shown anti-proliferative activity against other tumor cells, such as breast cancer cell line MDAMB-231, showing the ability to potentiate the breast cancer cell growth inhibition of 5-FU, a well-known cytostatic agent (Ramos et al., 2013). In general terms, the decrease of tumor cell viability by OMW has been mainly associated with the presence of Hydroxytyrosol and Hydroxytyrosol-derived compounds (Rosignoli et al., 2016; Rafehi et al., 2012). In addition, some of these compounds have been reported to synergize with conventional anti-cancer therapies (Fayyaz et al., 2016; Alipieva et al. 2014). There is extensive evidence of the environmental toxicity of many of these phenolic compounds present in OMW (Justino et al., 2012). However, the practical

implications of its toxic effect on human non-tumor cells are unknown and difficult to predict. This is mainly because bioavailability is unknown when these phenolic compounds reach the different organs and tissues. In addition, these compounds can be modified during the intestinal transit (Pinto et al., 2011). Further studies are needed to explore the bioavailability of the phenolic and secoiridoid compounds present in OMW and their role in inhibiting cell viability as well as their potential toxicity.

4. Conclusions

OMW could be used as a potential source of phenolic and secoiridoid compounds useful for controlling *Campylobacter*, the main food-borne pathogen, contributing to the revalorisation of olive oil by-products. Moreover, the potential of the bactericidal fraction as an anti-inflammatory agent was demonstrated as it markedly inhibited the production of NO in LPS-induced macrophages. These findings suggest that OMW can be an abundant and economically advantageous source of phenolic and secoiridoid compounds with antibacterial and anti-inflammatory properties, which might attenuate the infection and inflammation process induced by *Campylobacter*. Furthermore, in addition to therapeutic alternatives, bioactive compounds extracted from OMW would be potentially applicable in the different stages of the food chain where *Campylobacter* is present, as in the case of the production and processing of poultry meat, or as an alternative to disinfectants in the *Campylobacter* biofilm control. In all cases, to obtain the desired enrichment of phenolics and secoiridoids, and subsequently the desired antibacterial and anti-inflammatory activity, at least two purification steps are needed: microfiltration and solid phase extraction.

Acknowledgements

This work was founded through Project AGL2013-47694-R from the Consejo Superior de Investigaciones Científicas (Spain). The authors gratefully acknowledge Dr. Juan Fernández-Bolaños from the Instituto de la Grasa (CSIC) (Sevilla) for supplying purified 3,4-DHPG reference substance and Dr. Jordi Rovira (Universidad de Burgos, Spain) for his support with *Campylobacter* strains isolation.

References

- Aissa, I., Kharrat, N., Aloui, F., Sellami, M., Bouaziz, M., & Gargouri, Y. (2017). Valorization of antioxidants extracted from olive mill wastewater. *Biotechnology and Applied Biochemistry*, 64, 579–589.
- Alipieva, K., Korkina, L., Orhan, I. E., & Georgiev, M. I. (2014). Verbascoside-A review of its occurrence, (bio)synthesis and pharmacological significance. *Biotechnology Advances*, 32, 1065-1076.
- Aparicio-Soto, M., Sánchez-Fidalgo, S., González-Benjumea, A., Maya, I., Fernández-Bolaños, J. G., & Alarcón-de-la-Lastra, C. (2015). Naturally occurring hydroxytyrosol derivatives: hydroxytyrosyl acetate and 3,4-dihydroxyphenylglycol modulate inflammatory response in murine peritoneal macrophages. Potential utility as new dietary supplements. *Journal of Agricultural and Food Chemistry*, 63, 836-846.
- Araújo, M., Pimentel, F. B., Alves, R. C., & Oliveira, M. B. P. P. (2015). Phenolic compounds from olive mill wastes: Health effects, analytical approach and application as food antioxidants. *Trends in Food Science & Technology*, 45, 200-211.

- Bajouba, A., Pacchiarotta, T., Hurtado-Fernández, E., Olmo-García, L., García-Villalba, R., Fernández-Gutiérrez, A., Mayboroda, O.A. & Carrasco-Pancorbo, A. (2016). Comparing two metabolic profiling approaches (liquid chromatography and gas chromatography coupled to mass spectrometry) for extra-virgin olive oil phenolic compounds analysis: a botanical classification perspective. *Journal of Chromatography A*, 1428, 267–279.
- Bassani, B., Rossi, T., De Stefano, D., Pizzichini, D., Corradino, P., Macrì, N., Noonan, D. M., Albini, A., & Bruno, A. (2016). Potential chemopreventive activities of a polyphenol rich purified extract from olive mill wastewater on colon cancer cells. *Journal of Functional Foods*, 27, 236-248.
- Belaqziz, M., Tan, S. P., El-Abbassi, A., Kiai, H., Hafidi, A., O'Donovan, O., & McLoughlin, P. (2017). Assessment of the antioxidant and antibacterial activities of different olive processing wastewaters. *PLoS ONE*, 12, e0182622.
- Bernini, R., Gilardini Montani, M. S., Merendino, N., Romani, A., & Velotti, F. (2015). Hydroxytyrosol-derived compounds: a basis for the creation of new pharmacological agents for cancer prevention and therapy. *Journal of Medicinal Chemistry*, 58, 9089-9107.
- Bisignano, C., Filocamo, A., Ginestra, G., Giofre', S. V., Navarra, M., Romeo, R., & Mandalari, G. (2014). 3,4-DHPEA-EA from *Olea Europaea* L. is effective against standard and clinical isolates of *Staphylococcus* sp. *Annals of Clinical Microbiology and Antimicrobials*, 13, 24.
- Branciari, R., Ranucci, D., Ortenzi, R., Roila, R., Trabalza-Marinucci, M., Servili, M., Papa, P., Galarini, R., & Valiani, A. (2016). Dietary administration of olive mill

wastewater extract reduces *Campylobacter* spp. prevalence in broiler chickens. *Sustainability*, 8, 837.

- Bulotta, S., Oliverio, M., Russo, D., & Procopio, A. (2013). Biological activity of oleuropein and its derivatives. In K. G. Ramawat, & J. M. Merillon (Eds.), *Natural products* (pp. 3605-3638). Berlin: Heidelberg Springer-Verlag.
- Capasso, R., de Martino, A., & Arienzo, M. (2002). Recovery and characterization of the metal polymeric organic fraction (Polymerin) from olive oil mill wastewaters. *Journal of Agricultural and Food Chemistry*, 50, 2846-2855.
- Casas-Sanchez, J., Ansuncion, A. M., Herrlein, K. M., & Mestres, C. (2007). Interaction between the antibacterial compound, oleuropein, and model membranes. *Colloid and Polymer Science*, 285, 1351-1360.
- Castillo, S., Dávila-Aviña, J., Heredia, N., & Garcia, S. (2017). Antioxidant activity and influence of *Citrus* byproduct extracts on adherence and invasion of *Campylobacter jejuni* and on the relative expression of *cadF* and *ciaB*. *Food Science and Biotechnology*, 26, 453-459.
- Chowdhury, A. M. M. B., Akrotos, C. S., Vayenas, D. V., & Pavlou, S. (2013). Olive mill waste composting: A review. *International Biodeterioration & Biodegradation*, 85, 108-119.
- Cioffi, G., Pesca, M. S., De Caprariis, P., Braca, A., Severino, L., & De Tommasi, N. (2010). Phenolic compounds in olive oil and olive pomace from Cilento (Campania, Italy) and their antioxidant activity. *Food Chemistry*, 121, 105-111.
- Conforti, F., & Menichini, F. (2011). Foods of plant origin as source of nitric oxide production inhibitors. In S. Haugen, & S. Meijer (Eds.), *Handbook of nutritional*

biochemistry: Genomics, metabolomics, and food supply (pp. 385-403). Nova Science Publishers.

De Marco, E., Savarese, M., Paduano, A., & Sacchi, R. (2007). Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters. *Food Chemistry*, 104, 858-867.

Dermeche, S., Nadour, M., Larroche, C., Moulti-Mati, F., & Michaud, P. (2013). Olive mill wastes: biochemical characterizations and valorization strategies. *Process Biochemistry*, 48, 1532–1552.

ECDC (European Centre for Disease Prevention and Control), EFSA (European Food Safety Authority), and EMA (European Medicines Agency). (2017). ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals - Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) Report. *EFSA Journal*, 15, 4872.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). (2016). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. *EFSA Journal*, 14, 4634.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). (2017). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2015. *EFSA Journal*, 15, 4694.

- Facciola, A., Riso, R., Avventuroso, E., Visalli, G., Delia, S. A., & Lagana, P. (2017). *Campylobacter*: from microbiology to prevention. *Journal of preventive medicine and hygiene*, 58, E79-E92.
- Fayyaz, S., Aydin, T., Cakir, A., Gasparri, M. L., Panici, P. B., & Farooqi, A. A. (2016). Oleuropein mediated targeting of signaling network in cancer. *Current Topics in Medicinal Chemistry*, 16, 2477-2483.
- Ganan, M., Carrascosa, A. V., & Martinez-Rodriguez, A. J. (2009). Antimicrobial activity of chitosan against *Campylobacter* spp. and other microorganisms and its mechanism of action. *Journal of Food Protection*, 72, 1735-1738.
- Ganan, M., Collins, M., Rastall, R., Hotchkiss, A. T., Chau, H. K., Carrascosa, A. V., & Martinez-Rodriguez, A. J. (2010). Inhibition by pectic oligosaccharides of the invasion of undifferentiated and differentiated Caco-2 cells by *Campylobacter jejuni*. *International Journal of Food Microbiology*, 137, 181-185.
- Ganan, M., Silván, J. M., Carrascosa, A. V., & Martínez-Rodríguez, A. J. (2012). Alternative strategies to use antibiotics or chemical products for controlling *Campylobacter* in the food chain. *Food Control*, 24, 6-14.
- Grases, F., Prieto, R. M., Fernández-Cabot, R. A., Costa-Bauzá, A., Sánchez, A. M., & Prodanov, M. (2015). Effect of consuming a grape seed supplement with abundant phenolic compounds on the oxidative status of healthy human volunteers. *Nutrition Journal*, 14, 94-101.
- Hamza, E., Kittl, S., & Kuhnert, P. (2017). Temporal induction of pro-inflammatory and regulatory cytokines in human peripheral blood mononuclear cells by *Campylobacter jejuni* and *Campylobacter coli*. *PLoS ONE*, 12, e0171350.

- Hayek, S. A., Gyawali, R., & Ibrahim, S. A. (2013). Antimicrobial natural products. In A. Méndez-Vilas (Ed.), *Microbial pathogens and strategies for combating them: science, technology and education* (pp. 910-921). Badajoz: Formatex Research Center Publishing, Badajoz, Spain.
- Hentati, O., Oliveira, V., Sena, C., Bouji, M. S., Wali, A., & Ksibi, M. (2016). Soil contamination with olive mill wastes negatively affects microbial communities, invertebrates and plants. *Ecotoxicology*, 25, 1500-1513.
- International Olive Council (IOC). Update of world statistical series on production, imports, exports and consumption. (2016). <http://www.internationaloliveoil.org>
- Jerman Klen, T. (2014). Olive fruit phenols in olive oil processing: the fate and antioxidant potential, *PhD thesis*, University of Nova Gorica, Slovenia.
- Jerman Klen, T., & Mozetič Vodopivec, B. (2012). The fate of olive fruit phenols during commercial olive oil processing: traditional press versus continuous two- and three-phase centrifuge. *LWT-Food Science and Technology*, 49, 267-274.
- Justino, C. I., Pereira, R., Freitas, A. C., Rocha-Santos, T. A., Panteleitchouk, T. S., & Duarte, A. C. (2012). Olive oil mill wastewaters before and after treatment: a critical review from the ecotoxicological point of view. *Ecotoxicology*, 21, 615-629.
- Kaakoush, N. O., Castaño-Rodríguez, N., Mitchell, H. M., & Man, S. M. (2015). Global epidemiology of *Campylobacter* infection. *Clinical Microbiology Reviews*, 28, 687-720.
- Leouifoudi, I., Harnafi, H., & Zyad, A. (2015). Olive mill waste extracts: polyphenols content, antioxidant, and antimicrobial activities. *Advances in Pharmacological Sciences Volume, Article ID 714138*.

- Louwen, R., & Hays, J. P. (2013). Is there an unrecognised role for *Campylobacter* infections in (chronic) inflammatory diseases?. *World Journal of Clinical Infectious Diseases*, 3, 58-69.
- Lozano-Sánchez, J., Giambanelli, E., Quirantes-Piné, R., Cerretani, L., Bendini, A., Segura-Carretero, A. & Fernández-Gutiérrez, A. (2011). Wastes generated during the storage of extra virgin olive oil as a natural source of phenolic compounds. *Journal of Agricultural and Food Chemistry*, 59, 11491-11500
- Medina, E., Brenes, M., Garcia, A., Romero, C. & De Castro, A. (2009). Bactericidal activity of glutaraldehyde-like compounds from olive products. *Journal of Food Protection*, 72, 2611-2614.
- Medina, E., De Castro, A., Romero, C., Ramirez, E., & Brenes, M. (2013). Effect of antimicrobial compounds from olive products on microorganisms related to health, food and agriculture. In: A Méndez-Vilas (Ed.), *Microbial pathogens and strategies for combating them: science, technology and education* (pp.1087-1094). Badajoz: Formatex Research Center Publishing, Badajoz, Spain.
- Mengíbar, M., Ganan, M., Miralles, B., Carrascosa, A.V., Martínez-Rodríguez, A.J., Peter, M.G., & Heras, A. (2011). Antibacterial activity of products of depolymerization of chitosans with lysozyme and chitosanase against *Campylobacter jejuni*. *Carbohydrate Polymers*, 84, 844-848.
- Michel, T., Khlif, I., Kanakis, P., Termentzi, A., Allouche, N. A., Halabalaki, M. & Skaltsounis, A. L. (2015). UHPLC-DAD-FLD and UHPLC-HRMS/MS based metabolic profiling and characterization of different *Olea europaea* organs of Koroneiki and Chetoui varieties. *Phytochemistry Letters*, 11, 424-439.

- Mingo, E., Silvan, J. M., & Martinez-Rodriguez, A. J. (2016). Selective antibacterial effect on *Campylobacter* of a winemaking waste extract (WWE) as a source of active phenolic compounds. *LWT - Food Science and Technology*, 68, 418-424.
- Morales, D., Smiderle, F. R., Jimenez Piris, A., Soler-Rivas, C., & Prodanov, M. (2018). Production of a β -D-glucan-rich extract from Shiitake mushrooms (*Lentinula edodes*) by an extraction/microfiltration/reverse osmosis (nanofiltration) pilot process. *Innovative Food Science and Emerging Technologies* (sent for publication).
- Mosele, J. I., Martín-Peláez, S., Macià, A., Farràs, M., Valls, R. M., Catalán, Ú., & Motilva, M. J. (2014). Faecal microbial metabolism of olive oil phenolic compounds: *In vitro* and *in vivo* approaches. *Molecular Nutrition and Food Research*, 58, 1809-1819.
- Obied, H. K., Bedgood, D. R., Prenzler, P. D., & Robards, K. (2007). Bioscreening of Australian olive mill waste water extracts: biophenol content, antioxidant, antimicrobial and molluscicidal activities. *Food and Chemical Toxicology*, 45, 1238-1248.
- O'Brien, S. J. (2017). The consequences of *Campylobacter* infection. *Current Opinion in Gastroenterology*, 33, 14-20.
- Olaimat, A. N., Fang, Y., & Holley, R. A. (2014). Inhibition of *Campylobacter jejuni* on fresh chicken breasts by κ -carrageenan/chitosan-based coatings containing allyl isothiocyanate or deodorized oriental mustard extract. *International Journal of Food Microbiology*, 187, 77-82.

- Pinto, J., Paiva-Martins, F., Corona, G., Debnam, E.S., Oruna-Concha, M.J., Vauzour, D., Gordon, M.H., & Spencer, J.P.E. (2011). Absorption and metabolism of olive oil secoiridoids in the small intestine. *British Journal of Nutrition*, 105, 1607-1618.
- Prodanov, M., Vacas, V., Hernández, T., Estrella, I., Amador, B., & Winterhalter, P. (2013). Chemical characterisation of Malvar grape seeds (*Vitis vinifera* L.) by ultrafiltration and RP-HPLC-PAD-MS. *Journal of Food Composition and Analysis*, 31, 284-292.
- Rafehi, H., Ververis, K., & Karagiannis, T. C. (2012). Mechanisms of action of phenolic compounds in olive. *Journal of Dietary Supplements*, 9, 96-109.
- Ramos, R., Santos, S. A. O., Guerra, A. R., Guerreiro, O., Felício, L., Jerónimo, E., Silvestre, A. J. D., Pascoal Neto, C., & Duarte, M. (2013). Valorization of olive mill residues: antioxidant and breast cancer antiproliferative activities of hydroxytyrosol-rich extracts derived from olive oil by-products. *Industrial Crops and Products*, 46, 359-368.
- Richard, N., Arnold, S., Hoeller, U., Kilpert, C., Wertz, K., & Schwager, J. (2011). Hydroxytyrosol is the major anti-inflammatory compound in aqueous olive extracts and impairs cytokine and chemokine production in macrophages. *Planta Medica*, 77, 1890-1897.
- Ríos, J. L., & Recio, M. C. (2005). Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology*, 100, 80-84.
- Roig, A., Cayuela, M. L., & Sánchez-Monedero, M. A. (2006). An overview on olive mill wastes and their valorisation methods. *Waste Management*, 26, 960-969.

- Rosignoli, P., Fuccelli, R., Fabiani, R., Servili, M., & Morozzi, G. (2013). Effect of olive oil phenols on the production of inflammatory mediators in freshly isolated human monocytes. *The Journal of Nutritional Biochemistry*, 24, 1513-1519.
- Rubio-Senent, F., Rodriguez-Gutierrez, G., Lama-Muñoz, A., & Fernandez-Bolaños, J. (2012). New phenolic compounds hydrothermally extracted from the olive oil by-product alperujo and their antioxidative activities. *Journal of Agricultural and Food Chemistry*, 60, 1175-1186.
- Ryu, S. J., Choi, H. S., Yoon, K. Y., Lee, O. H., Kim, K. J., & Lee, B. Y. (2015). Oleuropein suppresses LPS-induced inflammatory responses in RAW 264.7 cell and zebrafish. *Journal of Agricultural and Food Chemistry*, 63, 2098-2105.
- Salaheen, S., Tabashsum, Z., Gaspard, S., Dattilio, A., Tran, T. H., & Biswas, D. (2018). Reduced *Campylobacter jejuni* colonization in poultry gut with bioactive phenolics. *Food Control*, 84, 1-7.
- Sato, K., Mihara, Y., Kanai, K., Yamashita, Y., Kimura, Y., & Itoh, N. (2016). Tyrosol ameliorates lipopolysaccharide-induced ocular inflammation in rats via inhibition of nuclear factor (NF)- κ B activation. *The Journal of Veterinary Medical Science*, 78, 1429-1438.
- Serio, A., Chiarini, M., Tettamanti, E., & Paparella, A. (2010). Electronic paramagnetic resonance investigation of the activity of *Origanum vulgare* L. essential oil on *Listeria monocytogenes* membrane. *Letters in Applied Microbiology*, 51, 149-157.
- Silvan, J. M., Mingo, E., Hidalgo, M., de Pascual-Teresa, S., Carrascosa, A. V., & Martinez-Rodriguez, A. J. (2013). Antibacterial activity of a grape seed extract and its fractions against *Campylobacter* spp. *Food Control*, 29, 25-31.

- Silvan, J. M., Mingo, E., & Martinez-Rodriguez, A. J. (2017). Grape seed extract (GSE) modulates *Campylobacter* pro-inflammatory response in human intestinal epithelial cell lines. *Food and Agricultural Immunology*, 28, 739-753.
- Soler-Rivas, C., Espín, J. C., & Wichers, H. J. (2000). Oleuropein and related compounds. *Journal of the Science of Food and Agriculture*, 80, 1013-1023.
- Suárez, M., Romero, M. P., & Motilva, M. J. (2010). Development of a phenol-enriched olive oil with phenolic compounds from olive cake. *Journal of Agricultural and Food Chemistry*, 58, 10396-10403.
- Thielmann, J., Kohnen, S., & Hauser, C. (2017). Antimicrobial activity of *Olea europaea* Linné extracts and their applicability as natural food preservative agents. *International Journal of Food Microbiology*, 251, 48-66.
- Tiwari, B. K., Valdramidis, V. P., O'Donnell, C. P., Kasiviswanathan, M., Bourke, P. & Cullen, P. J. (2009). Application of natural antimicrobials for food preservation. *Journal of Agricultural and Food Chemistry*, 57, 5987-6000.
- Venturi, F., Sanmartin, C., Taglieri, I., Nari, A., Andrich, G., Terzuoli, E., Donnini, S., Nicolella, C., & Zinnai, A. (2017). Development of phenol-enriched olive oil with phenolic compounds extracted from wastewater produced by physical refining. *Nutrients*, 9 (8), 916.
- Veza, T., Algieri, F., Rodríguez-Nogales, A., Garrido-Mesa, J., Utrilla, M. P., Talhaoui, N., Gómez-Caravaca, A. M., Segura-Carretero, A., Rodríguez-Cabezas, M. E., Monteleone, G., & Gálvez, J. (2017). Immunomodulatory properties of *Olea europaea* leaf extract in intestinal inflammation. *Molecular Nutrition and Food Research*, 61, 1601066.

- Yangui, T., Sayadi, S., Rhouma, A., & Dhouib, A. (2010). Potential use of hydroxytyrosol-rich extract from olive mill wastewater as a biological fungicide against *Botrytis cinerea* in tomato. *Journal of Pest Science*, 83, 437-445.
- Zhang, X., Cao, J., & Zhong, L. (2009). Hydroxytyrosol inhibits pro-inflammatory cytokines, iNOS, and COX-2 expression in human monocytic cells. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 379, 581-586.
- Zinnai, A., Sanmartin, C., Taglieri, I., Andrich, G., & Venturi, F. (2016). Supercritical fluid extraction from microalgae with high content of LC-PUFAs. A case of study: Sc-CO₂ oil extraction from *Schizochytrium* sp. *Journal of Supercritical Fluids*, 116, 126-131.

Figure legends.

Figure 1. Olive mill wastewater (OMW) clarification and fractionation pathway.

Figure 2. Antibacterial activity of the clarified olive mill wastewater (COMW) and COMW fractions (F1-F5) against *C. jejuni* 11168 strain. Results represent the mean $\pm$ standard deviation of colony forming units (CFU)/mL (n=3). Bars marked with asterisk indicate significant differences by ANOVA test ($p < 0.05$) compared to the control growth. C: growth control (bacteria+ culture medium).

Figure 3. Effect of the F5 COMW fraction (0.01 mg/mL) on nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophage cells. The data represent the mean values $\pm$ standard deviation of three independent experiments. Bars marked with asterisk indicate significant differences by ANOVA test ($p \leq 0.05$) on NO production compared to cells treated with 10 μ g/mL LPS. C: NO production in RAW 264.7 macrophage cells; LPS: NO production in LPS-stimulated RAW 264.7 macrophage cells; F5: NO production in LPS-stimulated RAW 264.7 macrophage cells incubated with F5 fraction.

Figure 4. Effects of the F5 COMW fraction on the viability of human intestinal HT-29 and Caco-2 cells. The results are expressed as percentage of control and are represented by mean $\pm$ SD (n = 3). Bars marked with asterisk indicate significant differences by ANOVA test ($p \leq 0.05$) compared to the control group. C: control (cells without F5)

Table 1. *Campylobacter* strains used in the present study.

Isolation source	Specie	Strain designation	Isolation place
Chicken food chain (FC)	<i>C. jejuni</i>	FC1	Cloacal swab slaughterhouse (Farm 1)
		FC2	Defeathering machine (Farm 1)
		FC3	Dirty crate (Farm 2)
		FC4	Clean crate (Farm 2)
	<i>C. coli</i>	FC5	Hamburger retail
		FC6	Drumstick retail
		FC7	Cutting table (Farm 1)
		FC8	Clean mince machine (Farm 2)
Campylobacteriosis patients (C)	<i>C. jejuni</i>	C1	Pediatric unit
		C2	Pediatric unit
		C3	Pediatric unit
		C4	Digestive unit
	<i>C. coli</i>	C5	Pediatric unit
		C6	Internal medicine unit
		C7	Internal medicine unit
		C8	Digestive unit
		C9	Pediatric unit

Table 2. Antibacterial activity (mg/mL) of F5 olive mill wastewater (OMW) fraction against different *Campylobacter* (*C. jejuni* and *C. coli*) strains.

Strains	Fraction 5	
	MIC	MBC
FC1 (<i>C. jejuni</i>)	0.25	0.43
FC2 (<i>C. jejuni</i>)	0.43	0.43
FC3 (<i>C. jejuni</i>)	1.00	1.25
FC4 (<i>C. jejuni</i>)	1.00	1.25
FC5 (<i>C. coli</i>)	0.62	1.25
FC6 (<i>C. coli</i>)	0.87	1.50
FC7 (<i>C. coli</i>)	1.75	2.00
FC8 (<i>C. coli</i>)	0.43	0.87
C1 (<i>C. jejuni</i>)	1.00	1.50
C2 (<i>C. jejuni</i>)	0.43	1.00
C3 (<i>C. jejuni</i>)	1.00	1.75
C4 (<i>C. jejuni</i>)	1.00	1.25
C5 (<i>C. coli</i>)	1.50	1.75
C6 (<i>C. coli</i>)	1.25	1.50
C7 (<i>C. coli</i>)	1.50	1.75
C8 (<i>C. coli</i>)	1.25	1.25
C9 (<i>C. coli</i>)	1.50	1.75

Abbreviations: MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; FC, food chain strain; C, clinical strain.

Table 3. Main secoiridoides and phenolic compounds identified in clarified olive mill wastewater (COMW) and COMW fractions in order of their retention time, UV-VIS absorption maximum ($\lambda_{\max}$) and molecular and fragment ions. Retention time (R_t); glucoside (glu); rutinoside (rut); rhamnoside (rham,).

Compound	R_t	$\lambda_{\max}$	$[M-H]^-$	Product ions	Presence in fraction
	(min)	(nm)	(m/z)	(m/z)	
3,4-dihydroxyphenylglycol (3,4-DHPG)	6.3	228/282	169.1	151.1	COMW/F2/F5
Hydroxytyrosol (3,4-DHPE)	16.0	227/280	153.1	123.1	COMW /F2/F5
Hydroxytyrosol glucoside (3,4-DHPE-glu)	18.2	228/278	315.0	153.1	COMW /F2/F5
Acyclodihydroelenolic acid glucoside (ADH-EA-glu)	21.9	233	407.1	-	COMW /F2/F5
Tyrosol (4-HPE)	23.0	228/277	137.1	-	COMW /F2/F5
Tyrosol glucoside (4-HPE-glu)	24.4	227/276	299.0	137.0	COMW /F2/F5
Demethyl elenolic acid glucoside (DMEA-glu or Oleoside)	33.7	240	389.0	-	COMW /F2/F5
Elenolic acid glucoside (EA-glu)	40.5	240	403.0	223.0, 179.0	COMW /F1/F2/F5
Hydroxytyrosol hydroxydecarboxymethyl elenolate (3,4-DHPE-HDCM-EA)	51.4	231/280	335.0	183.0, 151.0	COMW /F2/F5
β -Hydroxyverbascoside (1)	55.3	241/290sh/330	639.0	-	COMW /F2/F5
β -Hydroxyverbascoside (2)	56.1	241/290sh/330	639.0	-	COMW /F2/F5
Elenoic acid (form 1) (EA1)	61.9	238	241.0	139.1, 127.2, 101.2	COMW /F1/F2/F5
Quercetin-3-O-rut	63.7	238/266/356	609.0	-	COMW /F2/F5
Elenoic acid (form 2) (EA2)	66.0	238	240.9	209.1, 139.1, 127.1, 121.1, 101.1	COMW /F2/F5
Luteolin-7-O-glu	69.4	237/266/352	447.0	-	COMW /F2/F5

Verbascoside (3,4-DHPE caffeoyl-glu-rham)	69.8	239/288sh/334	623.0	293.1	COMW /F2/F5
Hydroxytyrosol decarboxymethyl dialdehyde elenolic acid (3,4-DHPE-DCM-EDA) (Oleacein)	82.4	232/281	319.0	195.1, 183.1	COMW /F2/F5
Oleuropein (3,4-DHPE-EA-glu)	86.0	233/280	539.1	377.1	COMW /F2/F5

Table 4. Content (mg/g of dry matter) of the main phenolics and secoiridoid derivatives identified in clarified olive mill wastewater (COMW) and COMW fractions. MV $\pm$ SD, mean value $\pm$ standard deviation (n =3).

Compound	COMW	F2	F5
	(mg/g) MV $\pm$ SD	(mg/g) MV $\pm$ SD	(mg/g) MV $\pm$ SD
3,4-dihydroxyphenylglycol (3,4-DHPG)	5.60 _a $\pm$ 0.19	5.22 _a $\pm$ 0.20	6.51 _b $\pm$ 0.20
Hydroxytyrosol (3,4-DHPE)	6.49 _b $\pm$ 0.29	5.84 _a $\pm$ 0.24	24.40 _c $\pm$ 0.95
Hydroxytyrosol glucoside (3,4-DHPE-glu)	8.40 _a $\pm$ 0.10	8.52 _a $\pm$ 0.13	26.80 _b $\pm$ 0.36
Tyrosol (4-HPE)	1.05 _a $\pm$ 0.04	2.95 _c $\pm$ 0.09	2.10 _b $\pm$ 0.04
Tyrosol glucoside (4-HPE-glu)	18.90 _b $\pm$ 0.80	16.11 _a $\pm$ 0.75	59.12 _c $\pm$ 1.88
Hydroxytyrosol hydroxydecarboxymethyl elenolate (3,4-DHPE-HDCM-EA)	2.00 _a $\pm$ 0.29	2.10 _a $\pm$ 0.09	5.15 _b $\pm$ 0.18
Quercetin-3-O-rut	0.50 _a $\pm$ 0.02	0.46 _a $\pm$ 0.02	2.16 _b $\pm$ 0.9
Luteolin-7-O-glu	28.22 _b $\pm$ 0.25	25.61 _a $\pm$ 0.11	55.27 _c $\pm$ 1.17
Hydroxytyrosol decarboxymethyl dialdehyde elenolic acid (3,4-DHPE-DCM-EDA) (Oleacein)	21.06 _b $\pm$ 0.96	18.59 _a $\pm$ 0.78	92.68 _c $\pm$ 1.99
Oleuropein (3,4-DHPE-EA-glu)	2.51 _a $\pm$ 0.08	2.89 _b $\pm$ 0.08	8.28 _c $\pm$ 0.36
Total phenol and secoiridoid derivatives	94.74	88.22	282.47

a, b, c, - Same letter in the same row indicates absence of significant differences (p < 0.05)

Highlights

- 1- OMW is a potential source of bioactive compounds for controlling *Campylobacter*
- 2- OMW is separated in 5 fractions by a hybrid MF/UF/SPE process
- 3- F5 fraction possesses antibacterial activity against *Campylobacter*
- 4- F5 fraction reduces LPS-mediated inflammation process
- 5- Antibacterial F5 fraction is rich in phenolic and secoiridoid compounds
- 6- OMW bioactivities increases the valorization of olive oil by-products

OMW - Olive mill wastewater

COMW - clarified OMW

MF - Microfiltration

UF - Ultrafiltration

DS - Dense suspension

F - Fraction

Figure 1

Figure 2

Figure 3

Figure 4