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ANTIMICROBIAL AND ANTI-BIOFILM ACTIVITY OF OLIVE OIL BY-PRODUCTS AGAINST *CAMPYLOBACTER* SPP. ISOLATED FROM CHICKEN MEAT

Rossana Roila[1], David Ranucci[1], Andrea Valiani[2], Roberta Galarini[2], Maurizio Servili[3], Raffaella Branciari[1]✉

[1]Dipartimento di Medicina Veterinaria, Università degli Studi di Perugia
Via San Costanzo 4, 06126 Perugia (PG), **Italy**
[2]Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati"
Via Salvemini 1, 06126 Perugia (PG), **Italy**
[3]Dipartimento di Scienze Agrarie, Alimentari ed Ambientali
Borgo XX Giugno 74, 06121 Perugia, (PG), **Italy**

ABSTRACT

Background. Worldwide, poultry is considered the main source of food-related human campylobacteriosis, which is generally associated with the consumption of raw or undercooked chicken meat. Furthermore, *Campylobacter* develops biofilms that are resistant to environmental stress, antibiotics, and disinfectants and are becoming a major issue for the food industry, especially the poultry industry. This study investigated the antimicrobial and anti-biofilm properties of polyphenols found in spray-dried olive mill wastewater (OMWW-SD) against *Campylobacter* strains isolated from chicken meat.

Material and methods. OMWW-SD was produced by dehydration of olive mill wastewater polyphenolic extract. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for OMWW-SD were determined by microdilution method whereas the inhibitory effect of the OMWW-SD on biofilm formation and biofilm disaggregation was tested through crystal violet assay on polystyrene plates.

Results. The phenolic profile of OMWW-SD mainly consisted of secoiridoid and hydroxycinnamic acid derivatives. Oleuropein-aglycone di-aldehyde (a secoiridoid derivative) was the major constituent, representing 72.5% of the total identified phenolic compounds. OMWW-SD showed a MIC ranging from 0.15 mg/mL to 0.3 mg/mL and a MBC of 0.3 mg/mL for all *Campylobacter* strains tested. The olive by-product extract tested was able, *in vitro*, to inhibit biofilm formation and to promote biofilm dispersion even at sub-MIC concentrations, with values ranging from 6% to 92% and from 4% to 83% at varying extract dilutions, respectively.

Conclusion. OMWW-SD could be developed as a new anti-biofilm agent with potential to control *Campylobacter* in the food chain, especially in the poultry industry, thereby enhancing food safety.

Keywords: *Campylobacter coli*, *Campylobacter jejuni*, minimum inhibitory concentration, minimum bactericidal concentration, phenolic compounds

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✉raffaella.branciari@unipg.it, phone 39 075 585 7936

INTRODUCTION

In 2016 campylobacteriosis was the most commonly reported zoonosis in Europe, as it had been since 2005, representing almost 70% of all the reported cases (EFSA/ECDC, 2017). Poultry is considered the main source of food-related human campylobacteriosis, which is generally associated with the consumption of raw or undercooked chicken meat and cross-contamination during raw chicken meat handling (Corry and Atabay, 2001; EFSA/ECDC, 2017). This is due to the fact that chickens, and other avian species, serve as natural reservoir hosts for thermophilic *Campylobacter* species and such bacteria are able to colonise their intestinal tract (Silva et al., 2011). Among the 14 species of the *Campylobacter* genus that are related to human disease, *Campylobacter coli* and *C. jejuni* are responsible for more than 95% of human campylobacteriosis (Fitzgerald, 2015). New concerns have now arisen due to the increase in incidence of infection caused by antibiotic resistant strains of *Campylobacter*, making this illness more difficult to treat (Zhang and Plummer, 2008). Furthermore, it has been reported that *Campylobacter* might develop biofilms that are more resistant than planktonic cells to environmental stress, antibiotics, and disinfectants and are thus becoming a major issue for the food industry, especially the poultry industry, and consequently for human health (Srey et al., 2013). A large proportion of European chicken production is contaminated with *Campylobacter* (EFSA/ECDC, 2017) and since poultry meat is highly perishable and consumption is growing globally (Daniel et al., 2011; Roila et al., 2018), its microbiological safety is of the utmost importance. Consumer concern about food safety has increased and, in this regard, there is growing interest in the use of natural antibacterial compounds like plant extracts rich in phenols (Artini et al., 2018; Silván et al., 2013). Such compounds have been demonstrated to exhibit antimicrobial properties and could represent an innovative approach to providing consumers with safer food products in the post-antibiotic era. Among natural products, olive-mill wastewater (OMWW), a by-product of the olive oil extraction process, could provide an alternative source of biologically active phenolic compounds that can be used in the food industry. Major phenolic compounds contained in olive oil by-products are included in the chemical classes of phenolic alcohols, secoiridoids derivatives, derivatives of hydroxycinnamic acid, phenolic acids, flavones, and lignans (Branciari et al., 2017). Phenols have already been reported to inhibit or delay the rate of growth of a range of bacteria and fungi (Artini et al., 2012; Pereira et al., 2006; Roila et al., 2016) and it has been demonstrated, *in vivo*, that feeding the birds phenolic extracts from olive mill wastewater resulted in a reduction in the prevalence of *Campylobacter* spp. in chickens (Branciari et al., 2016). Furthermore several studies in literature demonstrate the absence of toxicity of the OMWW phenolic compounds showing, instead, that these molecules are highly bioavailable and safe, indeed showing benefit effects on human health (Angelino et al., 2011; Fabiani et al., 2008; Soni et al., 2006; Zbakh and El Abbassi, 2012). The aim of the present study was to assess the antimicrobial and anti-biofilm activity of a polyphenolic extract (PE) from spray-dried olive mill wastewater (OMWW-SD) against strains of *C. jejuni* and *C. coli*.

MATERIALS AND METHODS

Antibacterial agent preparation and determination of phenolic composition

The spray-dried PE was obtained through dehydration, as described in Servili et al. (2011). In order to increase the stability of the PE, this was first combined with maltodextrins (1:1 w/w), then the slurry was subjected to dehydration with a spray dryer set to the following parameters: incoming air temperature of 180°C, outgoing air temperature of 80°C and turbine speed of 21,000 rpm. Five phenolic compounds were determined: tyrosol (p-HPEA), hydroxytyrosol (3,4-DHPEA), oleuropein-aglycone dialdehyde (3,4-DHPEA-EDA), verbascoside and pinoresinol. The analysis of OMWW-SD was conducted as follows. In a 50 mL polypropylene tube containing 1.0 g ±0.1 g of spray-dried sample, 25 mL of the extraction solution (methanol/water 80/20, v/v, with 20 mg/L of BHT) were added. The sample was shaken for 30 min and then centrifuged at 4500 rpm (10 min, 20°C). The pellet was re-extracted and the supernatants reunited and adjusted to a volume of 50 mL in a volumetric flask. Two dilutions (1 to 200 and 1 to 500) were prepared using the mixture Na-EDTA 0.1 M/MeOH

Table 1. Optimised MRM conditions for the analysis of the five phenolic compounds by LC-MS/MS

Analyte	RT, min	Adduct	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)
Hydroxytyrosol (3,4-DHPEA)	6.3	$[M-H]^-$	153.1	93.1	35
				95.1	28
				123.1	17
Tyrosol (*p*-HPEA)	8.2	$[M-H]^-$	137.1	106.1	18
				107.1	17
				119.1	18
				137.1	10
Verbascoside	12.1	$[M-H]^-$	623.2	135.1	48
				161.0	29
				342.1	37
Oleuperin-aglycone dialdehyde (3,4-DHPEA-EDA)	12.4	$[M-H+CH_3OH]^-$	351.1	165.1	15
				183.1	18
				195.1	13
				319.1	10
Pinoresinol	13.6	$[M-H]^-$	357.1	136.0	37
				151.0	21
				342.1	21

90/10 v/v and separately injected. The instrumental analysis was performed using a Surveyor MS pump (Thermo Finnigan, San Jose, CA, USA) coupled to a triple quadrupole mass analyser (TSQ Quantum Ultra, Thermo Finnigan) as previously described in Branciari et al. (2017). The separation was achieved on a Gemini C18 (100 × 2.0 mm, 3.0 µm, Penomenex, Torrance, CA, USA). The mobile phases were water (A) and methanol (B), the flow rate 0.25 mL/min and the injection volume 10 µL. The gradient started with 5% eluent B for 1 min, and linearly increased to 43% B in 8 min, followed by a linear increase to 95% B in 6 min. After 7 min, the system decreased to 5% B in 1 min and was re-equilibrated for 12 min (chromatographic run: 35 min). The column temperature was 30°C and the autosampler was thermostated at 16°C. The analyte ionisation was carried out using an electrospray source in negative mode (ESI-) with the MS analyser operating in MRM mode. Nitrogen was used as sheath (30 arbitrary units) and auxiliary gas (20 arbitrary units). The electrospray capillary voltage was set to –2.5 kV and the capillary temperature to 200°C. The selected MRM transitions and collision energies were listed in Table 1. Quantification was achieved by external standardisation using the more appropriate dilution (10,000 or 25,000 times on the whole) depending on the found concentration of each analyte.

Bacterial strains

Four *Campylobacter* spp. isolates were used in the current study: two were reference strains (*C. jejuni* ATCC 33291 and *C. coli* ATCC 33559) and two were chicken meat isolates (*C. coli* 40550 and *C. jejuni* 12054). The bacteria were stored in commercially prepared cryogenic Microbank vials at –80°C. Before the experiments the strains were sub-cultured in Preston

broth at 41.5°C under microaerobic conditions (85% N_2, 5% O_2 and 10% CO_2) to promote optimal growth.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for OMWW-SD were determined by the microdilution method in accordance with the standard VET01-A4 from Clinical Laboratory Standard Institute (CLSI, 2013) with slight modifications. Briefly, serial two-fold dilutions of spray-dried PE (from 0.075 mg/mL to 5 mg/mL of polyphenols) were prepared in 48-well microtitre plates in Müller Hinton Broth (MHB) supplemented with 5% defibrinated horse blood. Bacterial suspensions were prepared to a turbidity of 0.5 McFarland corresponding to an optical density of 0.125 at 620 nm and added to each well to a final concentration of 1×10^5 CFU/ml. Three controls were included in each microwell plate: antibiotic control (with erythromycin), organism control (with culture medium and bacterial suspension), and negative control (with culture broth and solution of polyphenols at the concentration tested). The plates were incubated at 41.5°C for 48 h under microaerobic conditions. After incubation, growth was assessed by plating the well contents on Agar blood plates, which were incubated at 41.5°C for 48 h under microaerobic conditions to promote optimal growth. The assay was repeated three times in triplicate.

The minimum inhibitory concentration – MIC was defined as the lowest concentration of tested compound able to maintain the suspension at the inoculum level after incubation (5 Log CFU/ml ±0.5 Log CFU/ml). MBC was defined as the lowest concentration of tested compound able to cause the death of 99.9% of the bacterial inoculum.

Anti-biofilm activity

In order to test the anti-biofilm activity of OMWW-SD, the inhibitory effect of the polyphenolic compound on biofilm formation and its activity on preformed biofilm were assayed. Biofilms were grown in 48-well flat bottom polystyrene microtitre plates according to the protocol described by Duarte et al. (2015), with some modifications. In brief, bacterial isolates were grown overnight in MHB supplemented with 5% of defibrinated horse blood at 41.5°C under microaerobic conditions and the turbidity of the inoculum suspension was adjusted to 0.5 McFarland corresponding to an optical density of 0.125 at 620 nm. Serial two-fold dilutions of OMWW-SD extract were prepared in culture medium (MHB 5% HB) directly into the plates, the final concentrations ranged from 0.25 × MIC to 4 × MIC. The bacterial suspension was added to the wells to a final concentration of 1×10^5CFU/ml and the plates were incubated at 41.5°C for 48 h in microaerobic conditions. In order to evaluate its effect on the dispersion of preformed biofilms the above-mentioned bacterial suspension was added into wells containing only MHB 5% HB and incubated for 48 h at 41.5°C in microaerobic conditions. After incubation, the contents of the well were removed, the OMWW-SD extract dilutions were added as mentioned above, and then the microtitre plate was incubated again for 48 h at 41.5°C. All the tests performed included positive controls (wells containing only bacterial suspension and culture medium) and negative controls (wells containing only culture medium).

Biofilm biomass was determined using a crystal violet staining method, as described by Reeser et al. (2007), with some modification. Briefly, following incubation the suspension culture was aspirated and the microwells washed with distilled water twice to remove loosely attached cells, then the plates were allowed to dry for 30 min at room temperature. 500 µL of 0.1% crystal violet (CV) solution was added to each well and kept for 30 min at room temperature, subsequently the CV solution was removed, the wells were washed twice with distilled water and then left to dry for 30 min at room temperature. The dye adhering to the microwells was dissolved with 95% ethanol (Sigma Aldrich), transferred into sterile disposable cuvettes, and read at 570 nm using a spectrophotometer (Ultrospec 2100 *pro*, Biochrom US). The experiment was repeated three times in triplicate.

Statistical analysis

The data were analyzed by repeated measures ANOVA, with *Campylobacter* strain and polyphenolic compounds concentration as fixed factors, using the GLM procedure of SAS (2001). Tukey post-hoc test was then performed, and differences were considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

Antibacterial agent phenolic composition

The phenolic composition of OMWW-SD is reported in Table 2. Among the five searched compounds, pinoresinol was not found (it was lower than the estimated limit of detection = 0.05 mg/g). The most abundant molecule was oleuropein-aglycone di-aldehyde (18.3 mg/g), followed by hydroxytyrosol (3.47 mg/g), verbascoside (3.09 mg/g) and tyrosol (0.39 mg/g). The composition of the PE found in the present study is similar to that reported by other authors. Roila et al. (2016) reported a phenolic profile of olive mill wastewater extract in which oleuropein-aglycone di-aldehyde was the major secoiridoid constituent (532.5 ±9.8 mg/g) followed by verbascoside (80.0 ±4.1 mg/g), hydroxytyrosol (56.5 ±1.1 mg/g) and tyrosol (12.3 ±0.4 mg/g). Servili et al. (2011) also reported for olive vegetation water concentrate, a phenolic profile (oleuropein-aglycone di-aldehyde – 16.9 g/L, verbascoside – 2.4 g/L, hydroxytyrosol – 0.03 g/L, tyrosol – 0.01 g/L) which resembled that tested against *Campylobacter* in this experiment. It has been reported that oleuropein-aglycone di-aldehyde exerts a wide range of bioactivities, as it is a strong antioxidant, anti-inflammatory, anticancer, and antimicrobial compound (Gill et al., 2005; Medina et al., 2006; Sindona et al., 2012).

Table 2. Phenolic profile of spray-dried olive mill wastewater, mg/g

Phenolic compound	Average value	Standard deviation
Hydroxytyrosol	3.47	0.20
Tyrosol	0.39	0.06
Oleuropein-aglycone di-aldehyde	18.27	1.03
Verbascoside	3.09	0.42
Total amount	25.2	

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The effect of olive mill wastewater PE on the growth of *C. coli* and *C. jejuni* was evaluated by the broth microdilution method; MIC and MBC experimental values are reported in Table 3. All the tested strains presented a MIC value of 0.15 mg/mL with the exception of *C. coli* 40550, which proved more resistant to the effect of OMWW-SD (MIC 0.3 mg/mL), while the MBC value was 0.3 mg/mL for all strains. In a recent experiment, Silvan et al. (2019) analysed the effects of olive mill wastewater fractions against *Campylobacter* spp. and reported an antibacterial activity (MIC from 0.25 to 1.75 mg/mL) in agreement with the present results. Furthermore, Šikić Pogačar et al. (2015) tested *Olea europea* leaf extract on *C. jejuni* growth: the leaf extract described by the authors had a phenolic composition similar to OMWW-SD with vesbascoside, oleuropein, and hydroxytyrosol being identified as its main polyphenolic constituents. Despite the similarity between the extracts, the MIC registered by Šikić Pogačar et al. (2015) for *C. jejuni* is considerably lower (1.25 µg/mL) than that reported in the present study. Although the antibacterial activity of the phenolic compounds derived from oil production wastes has already been evaluated *in vitro* for several microorganisms (Medina et al., 2006; Pereira et al., 2006; Roila et al., 2016; Serra et al., 2008; Tafesh et al., 2011), very few studies report testing olive-derived PEs on *Campylobacter* spp. Nevertheless, some authors have reported results concerning the effect of diverse natural extracts on the growth of *Campylobacter* spp. Sirirak and Voravuthikunchai (2011) tested *Eleutherine americana* essential oil against *Campylobacter* spp. isolated from chickens, finding a level of antibacterial activity (MIC 125–500 µL/mL) comparable to

Table 3. OMWW-SD Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) for two *Campylobacter* isolates (*C. jejuni* 12054 and *C. coli* 40550) and two reference strains (*C. jejuni* ATCC 33291 and *C. coli* ATCC 33559)

Campylobacter strains	MIC mg/mL	MBC mg/mL
Campylobacter coli ATCC 33559	0.15	0.3
Campylobacter coli 40550	0.3	0.3
Campylobacter jejuni ATCC 33291	0.15	0.3
Campylobacter jejuni 12054	0.15	0.3

that exerted by OMWW-SD in this study. Duarte et al. (2015) who tested resveratrol against *Campylobacter* spp. also found similar results (MIC 0.10 mg/mL). The same author tested the effect of coriander essential oil on *Campylobacter* spp. growth (Duarte et al., 2016), describing an antibacterial activity (MIC of 0.5–1 µL/mL) that was higher than that shown by OMWW-SD in this study; in addition, the author highlighted the perceptible antioxidant properties of the tested compounds, which were also found in this research (data not shown). Despite the recognised effect of diverse natural compounds on microbial growth, it is important to note that the above-mentioned effect is strongly determined by the composition of the plant extract, which varies greatly depending on the plant species analysed; moreover, even within the same plant species a certain degree of variability has been found. It has even been reported that for the olive (*O. europea*) the phenolic profile of olive drupes, leaves, and by-products can depend on several factors, such as the cultivar, the pedoclimatic conditions of the plant, and the oil extraction technique applied (Pereira et al., 2006).

Anti-biofilm activity

It is now recognised that biofilms are frequent source of infections (Costerton et al., 1999). Although the search for new and natural compounds that are able to effect antibacterial activity against planktonic cells of food borne pathogens has produced promising results, further effort needs to be made in order to find new strategies to inhibit or eliminate biofilm formation by these bacteria. Biofilms are highly-organised ecosystems, able to provide their inhabitants with a protective barrier from environmental stress and disinfectant. In particular, the microenvironment created within the biofilm may also protect *Campylobacter* cells from oxygen inactivation and increase the viability of cells (Buswell et al., 1998). Due to the above-mentioned resistance characteristics, pathogenic biofilms have been of considerable interest in the context of food safety (Shi et al., 2009) and *Campylobacter* biofilms have become especially problematic in poultry processing (Srey et al., 2013). In this study, concentrations of OMWW-SD ranging from 4 × MIC to 0.25 × MIC were tested on the formation (Fig. 1) and the dispersion (Fig. 2) of thermotolerant *C. coli* and *C. jejuni*

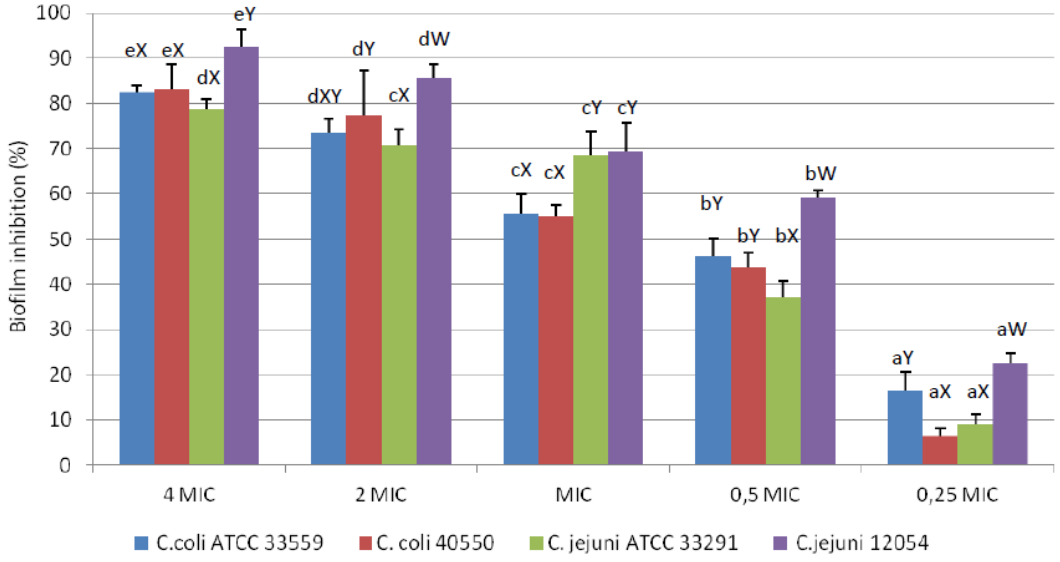

Fig. 1. Effects of different concentration of OMWW-SD on biofilm formation by *Campylobacter* spp. The results are expressed as percentage of biofilm inhibition: a, b, c, d, e – within each *Campylobacter* strain, different superscripts indicate differences between OMWW-SD concentrations ($p < 0.05$); X, Y, W, Z – within each OMWW-SD concentration, different superscripts indicate differences between *Campylobacter* strains ($p < 0.05$)

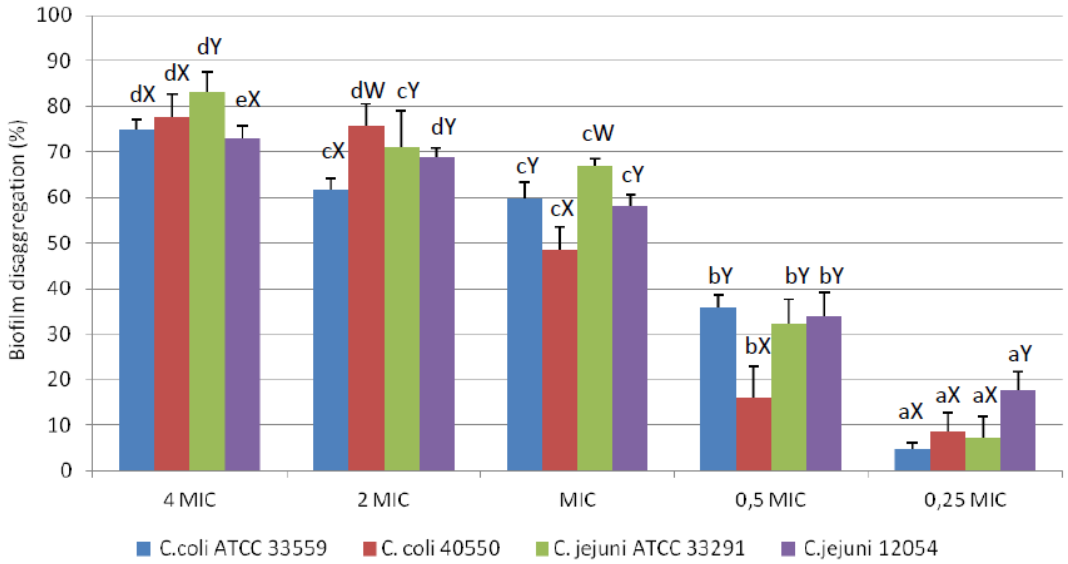

Fig. 2. Effects of different concentration of OMWW-SD on established *Campylobacter* spp. biofilm dispersion. The results are expressed as percentage of biofilm dispersion: a, b, c, d, e – within each *Campylobacter* strain, different superscripts indicate differences between OMWW-SD concentrations ($p < 0.05$); X, Y, W, Z – within each OMWW-SD concentration, different superscripts indicate differences between *Campylobacter* strains ($p < 0.05$)

biofilms. The effect of the phenolic extract on biofilm formation, as well as on established biofilm dispersion, both evaluated through CV assay, increased as the concentration of OMWW-SD increased. Concerning the activity of OMWW-SD on biofilm formation, when using the 4 × MIC concentration inhibition ranging from 79% to 92% of the total biofilm biomass was recorded, while at the lowest concentration employed (0.25 × MIC) the reduction in biofilm formation ranged from 6% to 23%. Among all strains, a different effect on biofilm formation was observed ($p \leq 0.05$) depending on the concentration of PE employed, with the exception of MIC and 2 × MIC for *C. jejuni* ATCC 33291. Concerning the results on the ability of OMWW-SD to disaggregate established biofilms (Fig. 2), the highest concentration tested (4 × MIC) exerted a dispersion activity ranging from 73% to 83% of the total biofilm biomass, whereas the lowest concentration tested (0.25 × MIC) revealed a reduction effect on preformed biofilm between 4% and 18%. Although, the effect on *Campylobacter* biofilm formation and disaggregation of OMWW-SD at concentration above MIC are most probably due to cells growth inhibition, the results confirm that OMWW-SD exerts an inhibitory effect on *Campylobacter* spp. biofilm formation, even when using the compound at concentrations below the MIC, in agreement with results already reported in the literature (Duarte et al., 2016; Szczepanski and Lipski, 2014). Furthermore, in line with the results of this research, Duarte et al. (2015; 2016) tested resveratrol and coriander oil on *Campylobacter* spp. biofilm, reporting an inhibition of biofilm formation ranging from 86% to 62% and from about 25% to less than 10%, and a biofilm mass dispersion ranging from 87% to 70% and from 40% to 10% for 4 × MIC and 0.25 × MIC concentrations, respectively. The effect of natural compounds on the biofilm produced by pathogenic microorganisms has already been reported: Artini et al. (2012) tested natural compounds derived from *Krameria*, *Aesculus hippocastanum* and *Chelidonium majus* reporting interesting antimicrobial and antibiofilm activity against *S. aureus* and *S. epidermidis*. Moreover, several essential oils have been tested against *P. aeruginosa* by Artini et al. (2018), showing that many of them were able to destabilize biofilm at very low concentration (48.8 µg/mL). Furthermore

Borges et al. (2012) tested two natural phenolic compounds (ferulic and gallic acid), demonstrating their potential to inhibit the biofilms of four important human pathogenic bacteria (*S. aureus*, *L. monocytogenes*, *E. coli* and *P. aeruginosa)*; these results are comparable to those of the present study, where biofilm formation reduction was around 70% for all the biofilms tested.

CONCLUSIONS

The results obtained in the present study confirm that olive mill wastewater extracts are able to limit the growth of *C. coli* and *C. jejuni*, to inhibit biofilm formation, and to disperse established biofilm. These findings suggest that OMWW-SD could be considered as a new natural anti-biofilm agent, highlighting its potential use to limit *Campylobacter* growth and biofilm formation in the food industry, especially in poultry processing, therefore enhancing food safety and limiting the use of chemical additives or preservatives.

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