Olive leaf extracts as a source of antibacterial compounds against *Campylobacter* spp. strains isolated from the chicken food chain

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INTRODUCTION

Campylobacter is the leading cause of bacterial foodborne gastroenteritis worldwide. Infections by *Campylobacter* in humans are generally caused by consuming contaminated foods of animal origin, with poultry, especially chicken, being the main reservoir. The high prevalence of *Campylobacter* in chicken carcasses and the growing resistance to the most widely used antibiotics has driven EFSA to propose a regulation (Regulation EU N° 2017/1495) containing new microbiological criteria to regulate the presence of *Campylobacter* in broiler carcasses (≤1,000 cfu/g). In this context, there has been an increase in the number of research aimed at the search for new tools to reduce *Campylobacter* incidence in chicken meat. The objective of the present work was to evaluate the antibacterial activity of two olive leaf extracts (OLE1 and OLE2) against eleven *Campylobacter* spp. strains (*C. jejuni* y *C. coli*) isolated from chicken food chain.

MATERIALS & METHODS

- **Preparation of Olive Leaf Extracts (OLEs)**
  - ✓ Drying olive leaves at 50°C for 30 min. (cabinet hot–air drier)
  - ✓ Milling dried olive leaves in a grinder
  - ✓ Extractions:
    - Aqueous extraction (60°C) → OLE1
    - Methanol extraction (40°C) → OLE2
  - ✓ Evaporation and freeze-drying of extracts

- **Phenolic composition of OLEs**
  - ✓ Analysis of phenolic compounds was performed by HPLC (Silvan et al., 2020)
- **Bacterial strains, growth media and culture conditions**
  - ✓ Eleven *Campylobacter* strains isolated from different points of the chicken food chain were used:

Campylobacter spp.

Species	Strain references
C. jejuni (7)	48, 222, 231, 262, 291, 321, 360
C. coli (4)	209, 270, 279, 577

VAIN workstation

  - ✓ Selective growth media: Brucella Broth (BB) and Mueller-Hinton agar supplemented with 5% sheep blood (MHB)
  - ✓ Growth conditions: strains reactivation into MHB and incubation for 48 h at 40°C, in microaerophilic atmosphere in VAIN workstation (85% N_2, 10% CO_2, 5% O_2)
- **Determination of antibiotic susceptibility**
  - ✓ Antibiotic susceptibility was performed by the *E-test* (Silvan et al., 2021)
  - ✓ Eight antibiotics were used:

- Erythromycin (ERY)	- Amoxicillin (AMX)
- Doxycycline (DOX)	- Amoxicillin-clavulanic acid (AMX-CLA)
- Ciprofloxacin (CIP)	- Gentamicin (GEN)
- Nalidixic acid (NAL)	- Tetracycline (TET)

- **Antibacterial activity**
  - ✓ Procedure:
    - 1 mL of extracts (2 mg/mL) disolved in BB (or BB for control growth)
    - 4 mL BB
    - 100 µL bacterial inoculum (~1x10^8 CFU/mL)
    - Incubation for 24 h at 40°C, 150 rpm, in microaerophilic atmosphere (VAIN)
    - Serial decimal dilutions of mixtures were plated onto fresh MHB agar
    - Incubation microaerobically for 72 h at 40°C in microaerophilic atmosphere (VAIN)
    - Antibacterial activity determination by CFU counting

Bacterial culture

RESULTS

Identification of olive leaves phenolic compounds

Table 1. Main phenolic compounds identified from olive leaf extracts by HPLC.

OLE1	Retention time	OLE2	Retention time
Hydroxytyrosol	18 min	Lueolin-7-O-glucoside	75 min
Hydroxytyrosol-glucoside	21.5 min	Verbascoside	76 min
Tyrosol	27.5 min	Oleuropein	93.7 min
Tyrosol-glucoside	35 min		
Lueolin-7-O-glucoside	75 min		

Antibiotic susceptibility

Table 2. Antibiotic resistance and MIC profile of *Campylobacter* spp. strains.

Specie	Ref. strain	ERY	TET	DOX	CIP	NAL	AMX	AMX-CLA	GEN	Rate of resistance
C. jejuni	48	S (0.5)	R (32)	R (48)	R (>32)	R (>256)	I (16)	S (0.5)	S (0.25)	4/8 (50%)
	231	S (0.5)	R (32)	R (48)	R (>32)	R (>256)	R (32)	S (0.5)	S (0.38)	5/8 (62.5%)
	262	S (0.5)	R (>256)	R (>256)	R (>32)	R (>256)	R (>256)	S (0.38)	S (0.19)	5/8 (62.5%)
	222	S (0.5)	R (>256)	R (>256)	R (>32)	R (>256)	R (>256)	S (0.25)	S (0.19)	5/8 (62.5%)
	321	S (0.38)	R (128)	R (>256)	R (>32)	R (>256)	R (>256)	S (0.19)	S (0.38)	5/8 (62.5%)
	291	S (0.75)	R (>256)	R (>256)	R (>32)	R (>256)	R (96)	S (0.25)	S (0.25)	5/8 (62.5%)
	360	S (0.38)	R (24)	R (32)	R (>32)	R (>256)	R (24)	S (0.25)	S (0.125)	5/8 (62.5%)
C. coli	279	S (0.5)	R (3)	R (12)	R (>32)	R (>256)	S (2)	S (0.5)	S (0.5)	4/8 (50%)
	209	S (0.75)	R (32)	R (8)	R (>32)	R (>256)	S (3)	S (1)	S (0.75)	4/8 (50%)
	577	S (2)	R (>256)	R (>256)	R (>32)	R (>256)	I (8)	S (0.75)	S (0.5)	4/8 (62.5%)
	270	S (1)	R (>256)	R (>256)	R (>32)	R (>256)	I (6)	S (1)	S (0.5)	4/8 (50%)
Resistant strains		0/11 (0%)	11/11 (100%)	11/11 (100%)	11/11 (100%)	11/11 (100%)	6/11 (46,2%)	0/11 (0%)	0/11 (0%)	

The breakpoints were defined following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (v11.0) and French Society of Microbiology. R: Resistant; S: sensitive; I: intermediate.

Antibacterial activity against *Campylobacter* strains

Table 3. Antibacterial activity of OLEs on the viable counts of different *Campylobacter spp.* strains after 24h of treatment at 2 mg/mL. Results are expressed as log CFU/mL ± SD deviation (n=4).

Species	Ref. strain	Control growth	OLE1	OLE2
C. jejuni	48	9.62^a±0.04	1.48^b±0.00	8.13^c±0.09
	231	8.63^a±0.03	1.48^b±0.00	6.98^c±0.09
	262	8.50^a±0.06	2.67^b±0.14	6.86^c±0.10
	222	9.21^a±0.07	8.58^a±0.05	9.39^a±0.05
	291	9.37^a±0.06	8.93^a±0.11	9.40^a±0.06
	321	8.92^a±0.08	9.14^a±0.07	9.24^a±0.04
	360	9.31^a±0.06	3.99^b±0.04	8.08^c±0.04
C. coli	270	8.64^a±0.03	1.48^b±0.00	6.69^c±0.02
	577	9.32^a±0.05	1.48^b±0.00	6.83^c±0.06
	279	9.47^a±0.04	1.48^b±0.00	9.78^a±0.03
	209	9.16^a±0.09	1.48^b±0.00	8.01^c±0.07

$^{a, b, c}$ values of CFU/mL in the same row marked with different letters indicate significant differences by ANOVA post hoc LSD Tukey test ($p<0.05$).

* Colony forming unit (CFU), detection limit was 1.48 log CFU/mL (30 CFU per plate).

CONCLUSIONS

- ❑ The analytical method used in the present work made it possible to identify hydroxytyrosol as the major phenolic compound present in OLE1; and oleuropein as the main phenolic compound present in OLE2.
- ❑ The studied strains of *C. jejuni* and *C. coli* isolated from the chicken food chain showed a high rate of resistance (≥50%) to the most commonly antibiotics used for campilobacteriosis treatment.
- ❑ However, some of the antibiotics evaluated in this study, such as ERY, AMX-CLA and GEN, showed 100% efficacy against *C. jejuni* and *C. coli* strains.
- ❑ Both extracts, OLE1 and OLE2, showed antibacterial activity against *Campylobacter* strains and this activity was strain-dependent.
- ❑ OLE1 completely inhibited the bacterial growth of two *C. jejuni* strains (48 and 231 strains) and all *C. coli* strains following a 24h exposure to 2 mg/mL, suggesting a broad antibacterial activity.
- ❑ OLE2 showed moderate antibacterial activity (after 24h at 2 mg/mL) against seven of the eleven (7/11) tested strains (48, 231, 262, 360, 270, 577, and 209) reducing 1-2 log CFU/mL the bacterial growth compared with the controls growth.
- ❑ OLEs could be consider as a potential source of bioactive compounds used as alternative for *Campylobacter* control at different stages of the chicken food chain, and would be an interesting tool to produce extracts capable of reducing the incidence of *Campylobacter* spp. in broiler chickens and thus comply with the new regulation.
- ❑ Furthermore, the recycling of olive industry by-products could also contribute to its revalorization, reducing also the environmental impact.

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REFERENCES: Regulation EU N° 2017/1495; Silvan et al. (2020), *Foods, 9, 1370*; Silvan et al. (2021), *Antioxidants*, 10, 943.

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