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# Dietary Administration of Olive Mill Wastewater Extract Reduces *Campylobacter* spp. Prevalence in Broiler Chickens

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**Abstract:** Food wastes are sources of compounds that can be used as natural additives in the food and feed industry. The olive oil industry produces two main wastes: aqueous waste (olive mill wastewater) and solid waste (pomace or olive cake). These by-products are rich in phenols, which are antioxidant and antimicrobial compounds able to inhibit or delay the growth of several bacteria in vitro. The dietary effect of both olive mill wastewater polyphenolic extract (OMWPE) and dehydrated olive cake (DOC) on the prevalence of *Campylobacter* spp. in broiler chickens was investigated. A commercial basal diet was supplemented with either OMWPE- or DOC-enriched maize at two dosages (low: 16%; high: 33%). The prevalence of *Campylobacter* spp. shedding was evaluated at 21, 35, and 49 days of age. The prevalence of *Campylobacter* spp. differed among groups only at 49 days of age. Both OMWPE groups showed a lower ( $p < 0.05$ ) prevalence compared to the control group. The odds ratio evaluation showed that the higher dose of OMWPE reduced the possibility of shedding 11-fold compared to the control group ( $p < 0.001$ ). These results highlight the potential use of olive by-products against *Campylobacter* spp. in poultry.

**Keywords:** broiler; olive mill by-products; polyphenols; microbial shedding

## 1. Introduction

Food waste is a major concern, as serious environmental issues are posed and limited options are available. This problem is intensified by slow progress in the development of effective waste management strategies and by the lack of legislation to regulate specific food waste discharge. Nonetheless, food waste is a valuable source of compounds that can be used in industrial applications. Olive oil extraction generates substantial amounts of by-products: a solid residue (olive pomace or olive cake) and an aqueous phase (olive mill wastewater), both of which are potential pollutants. Both are currently treated as industrial wastes or used as combustible material, heavy-metal absorbers and biofuel feedstock [1,2]. However, these by-products are relatively rich in polyphenols. Polyphenols are bioactive molecules commonly used as antioxidants and antimicrobials in the food industry [3–5]. One important alternative is the utilization of by-products from olive tree culture and the olive oil industry as sources of nutrients for animals [6]. Furthermore, new technologies based on membrane

treatments of aqueous by-products have been used to recover the crude phenolic concentrate rich in secoiridoids, especially 3,4-DHPEA-EDA and verbascoside [7], which can be used in industrial and feed applications. The valorisation of polyphenols, extracted from olive mill wastewater or available in stone-dried pomaces, as supplements in animal diets appears interesting for the possible bioactive effects on meat quality and animal welfare [8–10].

However, studies on in vivo antimicrobial effects and animal health and foodborne pathogen colonization and shedding are scarce. *Campylobacter* spp. is the main bacteria responsible for foodborne outbreaks in humans through chicken meat consumption [11]. The aim of this study was to evaluate the effects of dietary supplementation with two olive mill by-products, olive mill wastewater polyphenol extract (OMWPE) and dehydrated olive cake (DOC), on the shedding of *Campylobacter* spp. in broilers.

## 2. Experimental Section

### 2.1. Experimental Design and Animal Management

The two supplements used in the present experiment were obtained from the processing of Italian cultivar *Moraiolo* of *Olea europaea* through the dehydration of olive cake obtained by oil mechanical extraction from stone olives (DOC): for the aqueous waste, the supplement was obtained through the use of a filtration system with progressive permeability membranes (OMWPE) [12]. Both olive mill by-products were used in the production of a grower diet for broilers, fed from 22 to 49 days of age. To facilitate the feed processing and allow a correct distribution of the supplement within the feed matrix, a maize meal enriched with either OMWPE or DOC (provided by a local feed plant) was used. Briefly, maize flour (600 µm particle size) was thoroughly mixed with either OMWPE or DOC in a 30:70 or 50:50 proportion, respectively, and then dried (12 h) in a forced air oven at 60 °C. The enriched meal was used in two concentrations: 16% (L-OMWPE and L-DOC) and 33% (H-OMWPE and H-DOC). The highest dosage was chosen on the basis of previous studies on the use of dietary polyphenols in poultry and taking into consideration the estimated polyphenol content of the diet before pelleting [13,14]. An excessive polyphenol intake may have pro-oxidant activity [9].

The control group was fed a non-supplemented basal diet. All diets were isoenergetic, isonitrogenous and provided similar amounts of structural carbohydrates (Table 1). Rations were formulated to meet National Research Council standards [15]. The total concentrations measured for polyphenols in feed were obtained adding the individual concentrations of four compounds: tyrosol, hydroxytyrosol, pinoresinol and verbascoside. The identification and quantification of each compound was carried out by liquid chromatography tandem mass spectrometry (LC-MS/MS) in negative electrospray ionization (ESI) mode [16]. The LC-MS/MS system consisted of a Finnigan Surveyor LC pump combined with a triple stage quadrupole (TSQ) Quantum Ultra (Thermo Scientific, San Jose, CA, USA). The chromatographic separation was achieved using a Gemini analytical column (100 mm × 2.0 mm, 3 µm, Phenomenex, Torrance, CA, USA) with methanol and water as mobile phases. Samples were prepared as follows: five grams of feed were extracted twice with a mixture methanol/water 80/20 (*v/v*) containing 20 mg/L of BHT (2 × 25 mL). Two aliquots of the extract, 1 mL and 100 µL, were both diluted to 5 mL with a mixture Na<sub>2</sub>EDTA 0.1 M/methanol 90/10 (*v/v*). After filtration, the two aliquots were injected separately in LC-MS/MS. The instrument linearity range was studied from 1 to 1000 ng/mL. The validation study was performed at four levels: 0.1, 0.5, 5 and 50 mg/kg (six replicates per level in two occasions: 48 experiments).

A total of 495 22-day-old female chicks (Ross 308) were randomly assigned to five experimental grower diets (control CTR, L-DOC, H-DOC, L-OMWPE, and H-OMWPE) with three replicates of 33 birds each. The same starter diet (Table 1) was used for all experimental groups up to 21 days of age. The birds were supplied with feed and water ad libitum during the whole length of the trial and raised in an experimental farm with environmental conditions similar to those commonly found in industrial poultry houses.

**Table 1.** Chemical composition of the experimental diets.

	Starter	Grower/Finisher				
		Control	L-DOC	H-DOC	L-OMWPE	H-OMWPE
Moisture %	10.10	9.65	10.19	10.17	9.64	9.72
Crude Protein %	22.24	19.56	19.46	19.19	20.09	19.54
NDF %	17.93	22.14	22.16	23.52	23.58	22.50
Starch %	38.59	41.40	41.48	40.39	40.67	41.30

L-DOC = concentrate containing 16% DOC-enriched maize meal; H-DOC = concentrate containing 33% DOC-enriched maize meal; L-OMWPE = concentrate containing 16% OMWPE-enriched maize meal; HOMWPE = concentrate containing 33% OMWPE-enriched maize meal.

During the experiment, faeces were collected by cloacal swab to evaluate the efficacy of the diet in controlling the spread of *Campylobacter* spp. The faecal samples were collected randomly from 15 chickens for each replicate at 21 (i.e., before starting the administration of the experimental diets), 35 and 49 days of age (age of slaughtering) and sent under refrigerated conditions ( $4 \pm 1$  °C) to the laboratory for microbiological analyses, which were performed within 2 h from the sampling. All animals were weighed at the beginning and at the end of the trial. Carcasses were weighed after chilling and the dressing percentage was calculated as the ratio between final live weight and carcass weight.

## 2.2. *Campylobacter* spp. Isolation and Identification

For *Campylobacter* spp. isolation, selective enrichment was performed by incubating each swab sample microaerobically in 10 mL of Preston broth (Oxoid, Basingstoke, UK) at  $41.5 \text{ °C} \pm 1 \text{ °C}$  for  $44 \text{ h} \pm 4 \text{ h}$ . One loopful of broth culture was then streaked onto selective medium MCCD Agar (Biolife Italian s.r.l., Milan, Italy) and Karmali Agar (Oxoid), incubated at  $41.5 \text{ °C} \pm 1 \text{ °C}$  for  $44 \text{ h} \pm 4 \text{ h}$  in microaerobic conditions. At the end of the incubation period, *Campylobacter* typical colonies were cultured on blood agar medium (5% sheep red blood cells) (Biolife) and incubated at  $41.5 \text{ °C} \pm 1 \text{ °C}$  for 24–48 h in microaerobic conditions. Starting from the pure isolated cultures, colonies were tested through a morphological and biochemical identification (Gram stain, catalase test, oxidase test). Samples were considered positive when colonies presented morphological characteristics typical of the genus *Campylobacter* and were positive to oxidase and catalase. Subsequently, DNA was extracted from these colonies, isolated and amplified using multiplex PCR by simultaneous amplification of the genes of different species of *Campylobacter* in a single PCR reaction (multiplex) by the use of specific primers for the gene coding for the 23S ribosomal RNA of *Campylobacter* species [17].

## 2.3. Statistical Analyses

The data obtained were used to calculate the prevalence of shedding in all groups. The obtained prevalence was analysed by ANOVA (Statview SAS Institute Inc., Cary, NC, USA) with diet and sampling time as fixed factors. In addition, using data obtained from samples taken at slaughtering, a chi-square test (WINPEPI: PEPI-for-Windows freeware) was also performed to directly compare each experimental group to the control group and the odds ratio (and its 95% confidence intervals) was calculated to determine the probability level of detecting shedding animals. Performance data were analysed by ANOVA with diet as the fixed factor. Difference between least square means were evaluated using the Tukey test. The differences were considered significant at  $p < 0.05$ .

## 3. Results and Discussion

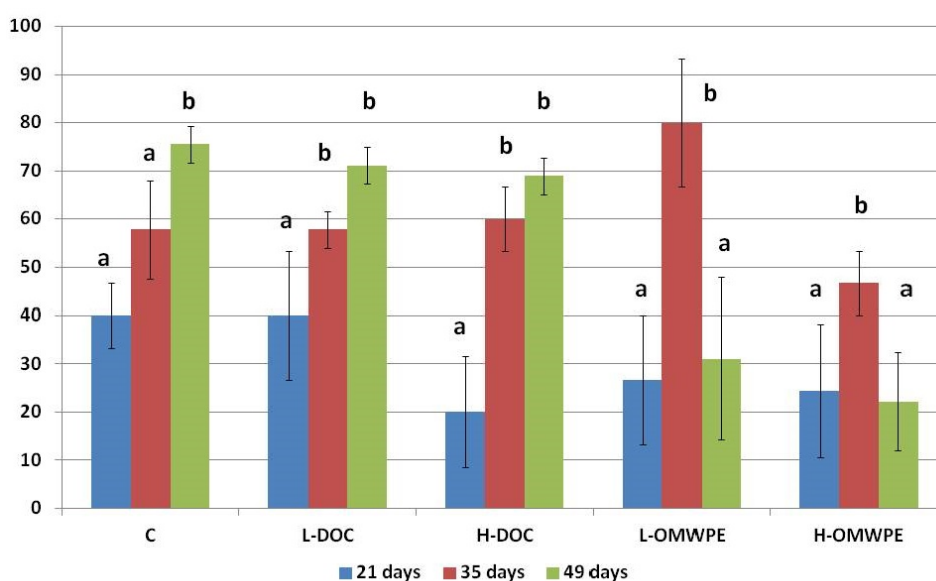
The five experimental diets contained the following amounts of polyphenols: control, 0.004 (g/kg); L-DOC, 0.014 (g/kg); H-DOC, 0.024 (g/kg); L-OMWPE, 0.263 (g/kg); H-OMWPE, 0.556 (g/kg). The prevalence of animal shedding *Campylobacter* spp. in the five groups considered is reported in Table 2. Even though the trials were performed under controlled conditions, inside each group

a relevant diffusion of *Campylobacter* spp. was detected as the shedding rate increased from 21 to 35 days of age in almost all the groups (Figure 1). An increasing in the shedding percentage was also noticed from 35 to 49 days, but only in the control and the L-DOC group, while a stable or a reduction trend was recorded in the other groups, with significant differences only observed for L-OMWPE and H-OMWPE vs. control ( $p < 0.01$ ). The high prevalence of *Campylobacter* spp. observed in the trial is considered typical in poultry flocks, as the rate of infection is relevant [18]. The high excretion rate generally provides, in a few days, a high degree of infection among the chickens of the same flock [19]. The use of various polyphenol doses seemed to affect the *Campylobacter* spp. shedding rate only after a prolonged period of administration (28 days).

**Table 2.** Average prevalence (%  $\pm$  standard deviation) of animal shedding *Campylobacter* spp. of control and the four trial groups at the three sampling periods considered.

	Control	L-DOC	H-DOC	L-OMWPE	H-OMWPE	<i>p</i> Value
21 days	40.00 $\pm$ 6.67	40.00 $\pm$ 13.33	20.00 $\pm$ 11.55	26.27 $\pm$ 13.33	24.44 $\pm$ 13.88	0.212
35 days	57.78 $\pm$ 10.18ab	57.78 $\pm$ 3.85ab	60.00 $\pm$ 6.67b	80.00 $\pm$ 13.33ab	46.67 $\pm$ 6.67a	0.012
49 days	75.56 $\pm$ 3.85b	71.11 $\pm$ 3.88b	68.89 $\pm$ 3.85b	31.11 $\pm$ 16.78a	20.00 $\pm$ 6.67a	<0.001

L-DOC = concentrate containing 16% DOC-enriched maize meal; H-DOC = concentrate containing 33% DOC-enriched maize meal; L-OMWPE = concentrate containing 16% OMWPE-enriched maize meal; HOMWPE = concentrate containing 33% OMWPE-enriched maize meal. ANOVA model. Different letters in the same row denote significant difference ( $p < 0.05$ ).



**Figure 1.** Average prevalence (%  $\pm$  standard deviation) of animal shedding of *Campylobacter* spp. at each of the three sampling periods. L-DOC = concentrate containing 16% DOC-enriched maize meal; H-DOC = concentrate containing 33% DOC-enriched maize meal; L-OMWPE = concentrate containing 16% OMWPE-enriched maize meal; H-OMWPE = concentrate containing 33% OMWPE-enriched maize meal. ANOVA Model. Different letters in the same row denote significant difference ( $p < 0.05$ ).

Previous studies have focused on the effects of olive by-product polyphenols on foodborne pathogens in in vitro trials [20,21], typically involving Gram-negative bacteria. No data are available on *Campylobacter* spp. both in in vitro and in vivo trials. Nonetheless, *Campylobacter* spp. seems to be sensitive to polyphenol compounds derived from other products [22]. The use of polyphenols from grape seed shows a strong capacity to inhibit *Campylobacter* spp. growth from 5 to 7 Log colony forming unit/g, and a minimal inhibitory concentration (MIC) of 20 mg/L and a minimal bactericidal concentration (MBC) of 60 mg/L against *Campylobacter jejuni* [23]. Furthermore, the MIC and MBC of blackberry pomace extract against *Campylobacter jejuni* were 0.6 mg/mL and 0.8 mg/mL Gallic

Acid Equivalent (GAE) respectively, whereas the MIC and MBC of blueberry pomace extract were 0.4 mg/mL and 0.5 mg/mL GAE [24]. The effect of a number of natural feed additives against *Campylobacter* spp. colonization in poultry has been previously studied by Kurecki et al. [25]. However, the used compounds were not comparable to the bioactive molecules considered in this study and the results were not consistent.

The higher amounts of polyphenols contained in the OMWPE diets are likely responsible for the observed effects on *Campylobacter* spp. shedding. The concentrations of polyphenols in poultry fed with maize meal enriched with dehydrated olive cake were too low to exert a significant action against the microorganisms.

The statistical analysis highlights the presence of differences between the control and OMWPE groups at 49 days, with a higher odds ratio for H-OMWPE (Table 3). A significant reduction of *Campylobacter* spp. faecal shedding was found for OMWPE diets only. These results suggest that, at the experimental conditions adopted, this polyphenol extract can be usefully included in broiler diets to control *Campylobacter* spp. shedding at the end of the production lifespan.

**Table 3.** Chi square test, odds ratio and confidence intervals of control group versus the other experimental group at 49 days of age.

	$\chi^2$	<i>p</i> Value	Odds Ratio	CI 95% Min	CI 95% Max
CTR v L-DOC	0.22	0.63	1.26	0.49	3.20
CTR v H-DOC	0.50	0.48	1.40	0.55	3.52
CTR v L-OMWPE	19.64	<0.001	7.61	2.98	19.42
CTR v H-OMWPE	25.61	<0.001	10.82	4.07	28.76

L-DOC = concentrate containing 16% DOC-enriched maize meal; H-DOC = concentrate containing 33% DOC-enriched maize meal; L-OMWPE = concentrate containing 16% OMWPE-enriched maize meal; H-OMWPE = concentrate containing 33% OMWPE-enriched maize meal.

As for the performance data reported in Table 4, the birds fed with H-DOC and L-OMWPE diets reached final live weights higher than those of the control animals ( $p < 0.05$ ). No differences were recorded among the other experimental groups. Higher carcass weight was observed for the L-OMWPE group compared to the CTR group. In contrast, no dietary effects on the dressing percentage were recorded. There is a dearth of data in the literature concerning the effect of olive mill by-products on broiler performance. To the best of our knowledge, DOC and OMWPE have not previously been used in broiler nutrition. Hughes et al. [26] used olive cake in broiler diets (7.2 g/kg) and observed lower weight gains in comparison to the control animals. Other results obtained after feeding broilers by-products rich in polyphenols are not consistent. Any comparison should be performed with caution because the feed characteristics and management practices adopted in these trials are extremely variable [14,27,28].

**Table 4.** Effect of dietary treatment on live weight, carcass weight, and dressing percentage.

	CTR	L-DOC	H-DOC	L-OMWPE	H-OMWPE	SE	<i>p</i> Value
<b>Live weight</b>	1.88b	2.02ab	2.17a	2.18a	1.94ab	0.059	0.017
<b>Carcass weight</b>	1.41b	1.51ab	1.58ab	1.64a	1.42b	0.053	0.045
<b>Dressing percentage</b>	74.71	74.41	72.69	75.28	73.13	0.726	0.134

L-DOC = concentrate containing 16% DOC-enriched maize meal; H-DOC = concentrate containing 33% DOC-enriched maize meal; L-OMWPE = concentrate containing 16% OMWPE-enriched maize meal; H-OMWPE = concentrate containing 33% OMWPE-enriched maize meal. SE = standard error of the mean. Different letters in the same row denote significant difference ( $p < 0.05$ ).

#### 4. Conclusions

The results obtained highlight the possible use of olive waste by-products in chicken diets to reduce the risk of *Campylobacter* spp. diffusion in the flock and consequently in meat. Further studies



are needed to evaluate whether dietary OMWPE administered throughout the chicken lifespan could also be used to prevent the colonization of *Campylobacter* spp. without any detrimental effect on performance. Finally, an improvement of feed technology to obtain DOCs with a higher polyphenol content should be explored with further research.

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**Author Contributions:** Andrea Valiani planned the study and coordinated the Istituto Zooprofilattico Sperimentale group composed by: Rossana Roila who collected the samples, Roberta Ortenzi and Paola Papa who performed the microbiological analysis, Roberta Galarini who performed polyphenol analysis in feeds, Massimo Trabalza-Marinucci who developed the chicken diet and performed the proximate composition of feed, Raffaella Branciarri who co-planned the study and wrote the manuscript, David Ranucci who collected data and performed a statistical evaluation. Maurizio Servili of the Department of Agricultural, Food and Environmental Sciences implemented the technology for the use of olive by-products as feed additives.

**Conflicts of Interest:** The authors declare no conflict of interest.

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