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Investigating the impact of supplementing drinking water with olive leaf extract on microbial load and oxidative stability of breast meat in broilers

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Abstract Deterioration of fresh meat quality is primarily attributed to microbial contamination and oxidative processes. Recently, plant-based natural preservatives, such as olive leaf extract (OLE), have gained considerable attention due to their antibacterial and antioxidant properties, which improve meat hygiene and prolong shelf life. This study evaluated the impact of supplementing broiler drinking water with ethanolic OLE on the oxidative stability and microbial load of breast meat in broilers. A completely randomized design was employed, comprising four treatment groups with four replicates of 20 birds each. Treatments included a control group (no OLE) and drinking water supplemented with 0.4%, 0.6%, or 0.8% ethanolic OLE for 42 days. After slaughter, breast meat samples were stored at 4°C for 12 days, during which lipid oxidation and bacterial counts were assessed every two days. Lipid oxidation was measured by determining malondialdehyde concentrations. Results showed that all OLE levels significantly reduced the *Enterobacter* counts up to day 12 compared to the control. The 0.6% OLE group exhibited the most pronounced antibacterial effect, reducing counts of *Enterobacter*, psychrotrophic bacteria, Lactobacillus, and total bacteria throughout the storage period. Additionally, malondialdehyde levels were consistently lower in all extract-treated samples, underscoring the antioxidant efficacy of OLE. In conclusion, supplementing broiler drinking water with OLE is an effective strategy to reduce bacterial contamination and enhance the oxidative stability of refrigerated breast meat, presenting a promising approach for improving poultry meat quality using plant-derived bioactive compounds.

Keywords: *Enterobacter*, hydroxytyrosol, *Lactobacillus*, oleuropein, psychrotrophic

Introduction

Chicken meat represents a primary source of dietary protein in Iran, and growing consumer awareness has intensified the demand for improved meat quality. Among the key factors compromising meat safety and shelf life are microbial contamination and lipid oxidation (Fletcher, 2002; Dominguez et al., 2019). From slaughter to consumption, poultry meat remains highly susceptible to bacterial contamination. While certain pathogens pose risks of foodborne illness, elevated microbial loads also contribute to spoilage, off-odors, undesirable flavors, and diminished nutritional value (Rouger et al., 2017). Therefore, effective preservation strategies targeting both microbial and oxidative deterioration are essential to maintain meat integrity.

Recent studies have highlighted the prevalence of microbial contamination in Iranian poultry products, underscoring the urgent need for targeted interventions (Ansarifar et al., 2023; Kiani Ghalesard et al., 2023). Various approaches are employed across the poultry production chain to mitigate contamination, including pre-slaughter and post-processing measures (Mortimore and Wallace, 2013; Marmion et al., 2021). Common practices involve rinsing with cold or warm water and applying disinfectants such as

chlorine, organic acids, chloramphenicol, sodium hypochlorite, and ammonium compounds to combat pathogens like *Enterobacter*, *Salmonella*, *Campylobacter*, *Listeria*, *Escherichia coli*, *Staphylococcus*, and *Pseudomonas* (Dickson and Anderson, 1992). Additional preservation techniques such as vacuum packaging, steam treatment, gamma irradiation, and chemical agents like hydrogen peroxide, ozone, peracetic acid, saponin, sodium bicarbonate, and trisodium phosphate have demonstrated efficacy in microbial control. Moreover, bioactive phytobiotics have emerged as promising alternatives for reducing bacterial contamination in poultry products (Milios et al., 2014).

Lipid oxidation presents another major challenge due to the high content of unsaturated fatty acids in poultry meat, which accelerates the formation of undesirable odors and toxic by-products (Papuc et al., 2017; Wu et al., 2024). This process, known as lipid peroxidation, involves free radical chain reactions that generate compounds such as malondialdehyde (MDA) (Dominguez et al., 2019). Although endogenous antioxidant systems, including the glutathione peroxidase pathway, offer some protection (Amaral et al., 2018), they may be insufficient to fully counteract oxidative stress, leading to quality degradation (Fu et al., 2022). Incorporating antioxidants is a widely accepted strategy to enhance oxidative stability (Wang et al., 2023). However, synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary-butylhydroquinone (TBHQ) have raised health concerns due to their potential toxicity and carcinogenicity (Ribeiro et al., 2019). Consequently, attention has shifted toward safer, natural alternatives particularly bioactive plant compounds such as olive leaf extract (OLE) for meat preservation (Bellucci et al., 2022).

The OLE is rich in phenolic compounds, including oleuropein and hydroxytyrosol, which exhibit potent antimicrobial and antioxidant activities, making it a promising candidate for natural meat preservation (Munekata et al., 2020; Saleh et al., 2020; Sánchez-Gutiérrez et al., 2021; Alesci et al., 2022). Supplementation of broiler diets with OLE has been shown to improve oxidative stability and reduce microbial load in breast meat stored at –18°C for six months (Da Silva et al., 2018). Additionally, immersing breast meat in ethanol-extracted OLE (0.25%, 0.50%, 1.0%) for 15 minutes and storing it at 4°C for 15 days significantly reduced psychrotrophic bacteria, *Enterobacter*, and *Staphylococcus* counts, along with lipid and protein oxidation (Saleh et al., 2020). These findings support the potential of OLE as a natural preservative in poultry meat.

Accordingly, the present study aimed to evaluate the efficacy of OLE as a biosafe additive in broiler drinking water to reduce microbial contamination and enhance oxidative stability in breast meat under refrigerated storage conditions.

Materials and methods

This study was conducted using a completely randomized design comprising of four treatment groups, each with four replicates of 20 Ross 308 broiler chicks, totaling 320 birds. The birds were housed in floor pens measuring 1.46 × 1.46 meters. The treatments included a control group (no OLE in drinking water) and groups receiving drinking water supplemented with 0.4%, 0.6%, or 0.8% ethanolic olive leaf extract (OLE) for 42 days. Starter, grower, and finisher diets were formulated according to Ross 308 nutritional guidelines (Aviagen, 2014). Although the nutrient composition of feed ingredients varied, all diets were adjusted to contain equivalent levels of energy and nutrients (Table 1).

The OLE was extracted by Zarband Pharmaceutical Company (Yasouj, Iran). At 42 days of age, one bird per replicate was slaughtered under sanitary conditions comparable to commercial standards. In a sterile laboratory environment, 12 breast muscle samples (2 g each) were collected from each bird. Six samples were used for bacterial enumeration, and six were allocated for oxidative stability analysis. All samples were stored at 4°C for 12 days, with assessments conducted on days 2, 4, 6, 8, 10, and 12 (Botsoglou et al., 2010).

Bacterial count assay

To prepare the peptone water solution for bacterial analysis, three grams of peptone powder (Merck, Darmstadt, Germany) were dissolved in 100 milliliters of distilled water. The solution was sterilized using an autoclave and stored under refrigeration until use. For sample processing, 500 microliters of the prepared solution were added to each tube containing a meat sample. From the initial suspension, 100 microliters were transferred to a second tube, and serial dilutions were performed up to the eighth dilution. The diluted samples were then inoculated onto selective agar media to enable enumeration of *Enterobacter*, psychrotrophic bacteria, *Lactobacillus*, and total bacterial count.

Each bacterial group was cultivated and quantified using specific media and incubation conditions. Violet Red Bile Agar (Merck, Darmstadt, Germany) was employed for *Enterobacter* enumeration, with samples incubated at 37°C for 48 hours. Psychrotrophic bacteria were grown on Plate Count Agar and incubated at 4°C for ten days. For *Lactobacillus*, MRS Agar (Merck, Darmstadt, Germany) was used, with incubation at 25°C for four days. Total bacterial counts were determined using Plate Count Agar with incubation at 25°C for 72 hours. Final bacterial counts were expressed as $\log_{10}$ colony-forming units (CFU) per gram of breast meat, following established methodologies (Davis, 2014; Foddai and Grant, 2020; Saravanan et al., 2021).

Oxidative stability

The oxidative stability of the meat samples was assessed by quantifying malondialdehyde (MDA), a principal secondary product of lipid peroxidation, using a thiobarbituric acid (TBA)-based spectrophotometric assay. To initiate the procedure, 8 mL of 10%

trichloroacetic acid (TCA) and 5 mL of a butylated hydroxytoluene (BHT) solution were added to each sample. The mixtures were homogenized at 18000 rpm for 1 minute using an Ultra-Turrax homogenizer (IKA Ultra-Turrax, Germany), and subsequently filtered through Whatman No. 42 filter paper. The resulting filtrate was brought to a final volume of 10 mL using TCA and centrifuged at 3000 rpm for 3 minutes (Hettich EBA 270, Tuttlingen, Germany). Following centrifugation, 2.5 mL of the supernatant was transferred to a clean test tube and mixed with an equal volume of TBA solution. The reaction was facilitated by incubating the samples in a water bath at 100°C for 35 minutes. After cooling to room temperature, absorbance was measured at a wavelength of 532 nm using a UV–Vis spectrophotometer (Unico UV-2100, USA). MDA concentrations were expressed in micrograms per gram of breast meat, providing a robust and reliable measure of lipid oxidation (Botsoglou et al., 1994; Papastergiadis et al., 2012).

Table 1. Percentage of dietary ingredients and nutrient composition of experimental diets across different time periods

Dietary Ingredients (%)	Days 1-10	Days 11-24	Days 25-42
Corn grain	51.48	53.26	58.34
Soybean meal	40.53	37.52	32.06
Vegetable oil	3.81	5.34	6.07
Dicalcium phosphate	1.58	1.43	1.26
Calcium carbonate	1.25	1.16	1.05
Salt	0.39	0.41	0.37
DL-Methionine	0.35	0.31	0.27
L-Lysine Hydrochloride	0.10	0.07	0.09
Vitamin premix*	0.25	0.25	0.25
Mineral premix*	0.25	0.25	0.25
Calculated nutrient composition			
Metabolizable energy (kcal/kg)	2859.87	2995.46	3091.79
Crude protein (%)	21.93	20.77	18.84
Calcium (%)	0.92	0.84	0.75
Available phosphorus (%)	0.46	0.42	0.38
Sodium (%)	0.18	0.18	0.16
Arginine (%)	1.41	1.33	1.19
Lysine (%)	1.37	1.25	1.11
Methionine + cysteine (%)	1.03	0.96	0.87
Threonine (%)	0.88	0.84	0.76
Tryptophan (%)	0.28	0.27	0.24

Vitamin and mineral premixes were added to each kilogram of the diet in the following amounts:
Vitamins (IU or mg): Vitamin A: 8640 IU, Vitamin D3: 1920 IU, Vitamin E: 17.28 mg, Vitamin K3: 1.92 mg, Thiamine: 1.68 mg, Riboflavin: 6.34 mg, Pantothenic Acid: 9.6 mg, Niacin: 28.8 mg, Pyridoxal Phosphate: 2.88 mg, Biotin: 0.96 mg, Vitamin B12: 0.0144 mg, Choline Chloride: 288 mg
Minerals (mg): Manganese: 95.232 mg, Zinc: 81.6 mg, Iron: 48 mg, Iodine: 0.96 mg, Copper: 9.6 mg, Selenium: 0.192 mg

Statistical analysis

Microbial culture and lipid oxidation data were log-transformed (base 10) prior to statistical analysis. Data were analyzed using the General Linear Model (GLM) procedure in SAS software (version 9.1.3; SAS Institute Inc., Cary, NC, USA), based on a completely randomized design and the following model:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where, Y_{ij} is the observed value, μ is the overall mean, T_i represents the treatment effect, and e_{ij} is the random error. Mean comparisons were performed using the Duncan's multiple range test at a 1% probability level.

Results

As shown in Table 2, supplementation of broiler drinking water with ethanol-extracted olive leaf (OLE) at concentrations of 0.4% to 0.8% significantly reduced *Enterobacter* counts in breast meat during the 12-day refrigeration period compared to the control group (P<0.01). However, the effect of increasing extract concentration did not follow a consistent pattern across all storage days. On days 2 and 4, the 0.8% treatment yielded lower *Enterobacter* counts than the 0.4% group. In contrast, on days 8, 10, and 12, the 0.6% treatment resulted in lower counts than the 0.8% group. No significant differences were observed between the 0.6% and 0.8% treatments on days 2, 4, and 6, indicating variability in antibacterial efficacy depending on storage duration.

Table 3 summarizes the effects of OLE treatments on *Lactobacillus* counts. While the 0.4% treatment showed a significant reduction only on day 4, both the 0.6% and 0.8% treatments significantly decreased *Lactobacillus* counts throughout the storage period. The 0.8% group exhibited lower counts than the 0.6% group on days 2, 4, 8, and 10.

Regarding psychrotrophic bacteria (Table 4), the 0.6% treatment significantly reduced bacterial counts across all storage days (P<0.01). The 0.4% treatment showed no significant effect on day 8, and the 0.8% treatment did not differ significantly from the control on days 6, 10, and 12.

The total bacterial counts were lower in the 0.4% to 0.8% extract treatments compared to the control group during the initial stages of refrigerated storage (days 2 to8). Notably, the 0.6% extract treatment demonstrated a consistently reduced bacterial load throughout the entire 12-day storage period. No statistically significant differences (P>0.01) were observed in the 0.4% treatment on days 10 and 12, nor in the 0.8% treatment on day 10, with regard to total bacterial counts (Table 5). These findings suggest that a concentration of 0.6% ethanol extract of olive leaves may be optimal for reducing microbial contamination and improving bacterial control in refrigerated broiler breast meat.

Table 2. Effects of olive leaf extract (OLE) supplemented to drinking water on the *Enterobacter* bacterial count (log_{10} cfu/g) in chicken breast meat

Treatment	Refrigeration storage days					
	2	4	6	8	10	12
Control (No OLE)	6.2^{a}	6.9^{a}	7.9^{a}	8.1^{a}	8.6^{a}	8.5^{a}
0.4% OLE	5.9^{b}	6.6^{b}	7.1^{b}	7.3^{bc}	7.7^{c}	7.7^{b}
0.6% OLE	5.6^{bc}	6.1^{c}	6.9^{b}	7.0^{c}	7.6^{c}	7.4^{c}
0.8% OLE	5.4^{c}	6.2^{c}	7.1^{b}	7.4^{b}	8.2^{b}	8.0^{b}
P Value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
SEM	0.03	0.03	0.03	0.02	0.05	0.04

$^{a, b, c}$: Within each column, means with common superscript are not different (P>0.05)

Table 3. Effects of olive leaf extract (OLE) supplemented to drinking water on the *Lactobacillus* bacterial count (log10 cfu/g) in chicken breast meat

Treatment	Refrigeration storage days					
	2	4	6	8	10	12
Control (No OLE)	6.4^{a}	7.1^{a}	8.1^{a}	8.4^{a}	8.4^{a}	7.5^{a}
0.4% OLE	6.2^{a}	6.8^{b}	7.9^{ab}	8.2^{a}	8.2^{ab}	7.4^{a}
0.6% OLE	5.8^{b}	6.6^{b}	7.5^{bc}	7.6^{b}	8.1^{b}	7.2^{b}
0.8% OLE	5.4^{c}	6.1^{c}	7.2^{c}	6.6^{c}	7.5^{c}	7.1^{b}
P Value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
SEM	0.04	0.02	0.05	0.06	0.02	0.02

$^{a, b, c}$: Within each column, means with common superscript are not different (P>0.05)

Table 4. Effects of olive leaf extract (OLE) supplemented to drinking water on the psychrotrophic bacterial count (log10 cfu/g) in chicken breast meat

Treatment	Refrigeration storage days					
	2	4	6	8	10	12
Control (No OLE)	5.2^{a}	5.8^{a}	6.3^{a}	6.6^{a}	7.0^{ab}	7.6^{a}
0.4% OLE	4.8^{b}	5.3^{b}	6.0^{b}	6.4^{ab}	6.8^{b}	7.0^{bc}
0.6% OLE	4.6^{b}	5.2^{b}	5.6^{c}	5.9^{c}	6.4^{c}	6.7^{c}
0.8% OLE	4.7^{b}	5.4^{b}	6.1^{ab}	6.2^{bc}	7.1^{a}	7.2^{ab}
P Value	<0.01	0.02	0.01	<0.01	<0.01	<0.01
SEM	0.02	0.03	0.03	0.04	0.02	0.04

$^{a, b, c}$: Within each column, means with common superscript are not different (P>0.05)

Table 5. Effects of olive leaf extract (OLE) supplemented to drinking water on the total bacterial count (log_{10} cfu/g) in chicken breast meat

Treatment	Refrigeration storage days					
	2	4	6	8	10	12
Control (No OLE)	6.3^{a}	7.1^{a}	7.9^{a}	8.8^{a}	8.8^{a}	9.2^{a}
0.4% OLE	6.1^{b}	6.9^{b}	7.7^{b}	8.4^{b}	8.6^{ab}	9.0^{ab}
0.6% OLE	5.9^{c}	6.7^{d}	7.4^{c}	8.1^{b}	8.3^{b}	8.4^{c}
0.8% OLE	6.1^{b}	6.9^{bc}	7.5^{c}	8.3^{b}	8.5^{ab}	8.7^{bc}
P Value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
SEM	0.01	0.01	0.01	0.02	0.04	0.05

$^{a, b, c}$: Within each column, means with common superscript are not different (P>0.05)

Table 6 presents the statistical comparison of mean malondialdehyde (MDA) levels in broiler breast meat, a key indicator of oxidative stability, across experimental treatments during the 12-day refrigeration period. Given that MDA is a byproduct of lipid peroxidation, lower levels indicate enhanced oxidative stability. The application of olive leaf ethanol extract at all tested concentrations resulted in improved oxidative stability compared to the control group, which did not receive the extract. Although the 0.8% extract treatment recorded the lowest MDA concentration on day 4, this effect was not consistently observed at other time points. Therefore, increasing the extract concentration from 0.4% to 0.8% did not yield a consistent or significant enhancement in oxidative stability. Notably, significant differences in MDA levels were observed among extract treatments on certain storage days, such as days 10 and 12 (Table 6), indicating that higher extract concentrations may offer occasional, though not uniform, benefits in mitigating lipid oxidation.

Discussion

The findings of this study emphasize the efficacy of incorporating ethanol extract of olive leaves (OLE) into

broiler drinking water as a safe and multifunctional additive aimed at enhancing meat quality. Notably, the significant reductions in microbial load and improvements in oxidative stability observed throughout the 12-days refrigerated storage period underscore the antibacterial and antioxidant properties of OLE in poultry production. Through its dual action in controlling bacterial contamination and inhibiting lipid oxidation, this plant- based compound offers a promising alternative to synthetic preservatives, thereby addressing key challenges related to meat safety and shelf life (Borjan et al., 2020). The antimicrobial activity of OLE contributes to suppressing spoilage-associated bacterial growth, effectively extending shelf life and preserving hygienic standards (Bellucci et al., 2022). Concurrently, its antioxidant capabilities help maintain muscle tissue integrity by preventing lipid peroxidation, which otherwise leads to unfavorable sensory attributes and nutritional deterioration (Dominguez et al., 2019). These combined effects affirm the practical relevance of OLE in meat preservation and support its broader implementation in commercial poultry operations.

Table 6. Effects of olive leaf extract (OLE) supplemented to drinking water on malondialdehyde* content in chicken breast meat (µg/g)

Treatment	Refrigeration storage days					
	2	4	6	8	10	12
Control (No OLE)	0.32^{a}	0.35^{a}	0.37^{a}	0.40^{a}	0.51^{a}	0.56^{a}
0.4% OLE	0.25^{b}	0.27^{b}	0.28^{c}	0.33^{bc}	0.39^{b}	0.43^{bc}
0.6% OLE	0.22^{cd}	0.26^{b}	0.28^{cd}	0.30^{bc}	0.31^{c}	0.38^{cd}
0.8% OLE	0.20^{d}	0.23^{c}	0.25^{d}	0.29^{c}	0.29^{c}	0.31^{d}
P Value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
SEM	0.02	0.02	0.02	0.05	0.09	0.10

$^{a, b, c}$: Within each column, means with common superscript are not different (P>0.05)
*Malondialdehyde concentration is inversely related to oxidative stability

In addition to meat quality improvements, previous investigations have reported physiological advantages of olive leaf phytobiotics, including enhanced intestinal functionality, balanced gut microbiota, improved digestive efficiency, and increased growth performance (Hadidi et al., 2022; Selim et al., 2022). Central bioactive constituents in OLE, particularly oleuropein and hydroxytyrosol, have also demonstrated immunomodulatory effects and protective roles against pathogens and inflammatory processes (Bisignano et al., 1999), further substantiating its value as a natural feed additive.

These results are in alignment with existing literature. For example, dietary supplementation with olive leaf powder in broilers has been associated with substantial reductions in *Escherichia coli* and *Salmonella* counts, along with enhanced oxidative stability in frozen chicken meat (Da Silva et al., 2018). Similarly, administering ethanol-extracted olive leaves at concentrations ranging from 0.1% to 0.5% has been shown to lower malondialdehyde levels in broiler breast meat, indicating improved oxidative stability (Xie et al., 2022). Although microbial load in meat was not assessed in that study, increases in beneficial bacterial populations such as *Lactobacillus* and *Bifidobacterium*, along with decreases in *E. coli* counts, were reported in cecal samples of treated birds. The water-based delivery method used in the present investigation may present advantages over feed-based approaches by enabling more uniform consumption, simplified application, and potentially enhanced bioavailability.

The antimicrobial and antioxidant effects observed in this study are largely attributed to the phenolic richness of OLE, specifically oleuropein and hydroxytyrosol (Sánchez-Gutiérrez et al., 2021), which are widely recognized for their bioactivity (Borjan et al., 2020; Xie et al., 2022). In vitro analyses confirm their effectiveness against a broad spectrum of bacterial species, with hydroxytyrosol exhibiting greater antimicrobial potency than oleuropein, despite its lower concentration in olive leaves (Bisignano et al., 1999).

Much of the prior research concerning OLE has centered on its impact on gut microbiota, showing increased populations of *Lactobacillus* and decreased levels of *E. coli* when administered via feed or water (Jalalfard et al., 2021; Vaziri et al., 2021). This study expands upon such findings by demonstrating that OLE supplementation in drinking water reduces a wider range of bacterial groups within the meat itself, including *Enterobacter*. Interestingly, *Lactobacillus* counts declined in refrigerated breast meat over the storage period, mirroring trends observed in other microbial populations. While Gram-negative bacteria such as *Enterobacter* and *Campylobacter* are well-established contributors to meat spoilage and foodborne diseases (Rouger et al., 2017), Gram-positive lactic acid bacteria may also adversely affect meat quality through metabolic activities that alter texture, flavor, and product stability (Pothakos et al., 2015).

Conclusions

The supplementation of broiler drinking water with varying concentrations of olive leaf extract (OLE) effectively reduced bacterial counts and improved the antioxidant capacity of breast meat during refrigerated storage, thereby highlighting its potential as a natural intervention for enhancing meat quality. In the light of OLE's demonstrated efficacy as both an antimicrobial and antioxidant agent, its application in poultry production represents an environmentally sustainable and viable alternative to conventional chemical preservatives. These findings align with the growing interest in plant-derived additives within health-

conscious and eco-conscious meat processing sectors. Future research should aim to identify the optimal concentration of OLE required to achieve targeted microbial control and oxidative stability, while also elucidating the underlying mechanisms of its protective effects and evaluating its scalability in commercial poultry production systems.

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Conflict of interest

The authors declare there is no conflict of interest regarding of this research.

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