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Protective Effect of Carnosic Acid against Contamination of Broiler Meat with *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

Due to its biological composition, which renders it extremely vulnerable to microbial deterioration, poultry meat is considered a highly perishable. A number of variables, including temperature, oxygen levels, enzyme activity, moisture content, light exposure, and microbial contamination, affect the quality and shelf life of fresh chicken meat. The use of natural preservatives as safe substitutes for artificial chemicals has drawn more attention in recent years. Eighty broiler breast and thigh samples were taken from several broiler farms in Basra City for analysis. This study used pure carnosic acid (98%) to extend the shelf life to prolong the shelf life of chicken meat stored in a refrigerator at 4 °C. The antibacterial efficacy of carnosic acid as a natural preservative was assessed in this study. In order to investigate the impact of supplemental CA on meat preservation, broiler chickens (breast and thigh) were treated with varying concentrations of acid (0.05, 0.10, and 0.15 g/kg of each flesh) and then stored for three, seven, and fourteen days. *Escherichia coli* and total bacteria were measured using traditional techniques. *Staphylococcus aureus* was detected using as morphological characteristics, physiological (coagulase tube method), classical methods biochemical tests, and growth on selective medium as Mannitol Salt Agar (MSA). Polymerase chain reaction (PCR) was performed to amplify the nuc gene in this isolated species, indicating the presence of nuc size (756) bp compared with a ladder used. According to this result the carnosic acid act as antibacterial agent and lower the counts of pathogenic bacteria.

1-Introduction

Chicken is one of the most widely consumed sources of animal protein worldwide due to its affordability, nutritional value, and versatility in preparation. However, the quality of chicken meat is influenced by several factors, including genotype, rearing system, and feeding practices, which can ultimately affect the technological and sensory characteristics of the final product [1]. Chicken meat is an essential component of a balanced diet, being rich in easily digestible protein (due to its low collagen content), essential amino acids, vitamins, minerals, and unsaturated fatty acids, thereby providing multiple health benefits for people of all ages [2].

Despite these nutritional advantages, fresh chicken meat is considered a highly perishable food product. Its high nutrient content, elevated water activity, and near-neutral pH create favorable conditions for the rapid growth of spoilage and pathogenic microorganisms, leading to a rapid loss of freshness and a short shelf life [3,4]. Microbial growth is therefore the primary factor responsible for the spoilage of refrigerated chicken meat [5]. The metabolic activities of bacteria produce secondary metabolites that alter the physicochemical and sensory attributes of the meat [6].

Over the years, various preservation strategies have been developed to extend the shelf life of poultry products. Nevertheless, the risk of recontamination remains a significant concern. Microbial contamination is one of the major challenges in the food industry, with serious implications for public health due to foodborne illnesses, as well as substantial economic losses caused by product spoilage and waste [7].

The microorganisms primarily responsible for spoilage in poultry meat are predominantly surface-associated. Microorganisms, predominantly *Staphylococcus aureus* and *Escherichia coli*, along with inherent biochemical reactions, lead to changes such as proteolysis, amino acid degradation, and, most importantly, lipid oxidation. These, in the final stages of poultry meat storage, induce the development of off-flavors and sliminess [8,9]. leading to a decline in quality and a shortened commercial shelf life [10]. The high incidence of foodborne pathogens makes food a common vector for disease, especially raw meat and meat products [11]. *Staphylococcus aureus*, a bacterium, can be spread through human contact during food preparation, processing, and cooking. Proper food handling and preservation are critical because the heat-stable toxins in *Staphylococcus aureus*-infected meat can cause widespread food poisoning [12]. Meat processing hygiene is essential because Enterobacteriaceae, including Salmonella, Escherichia E-coli, are found in meat and poultry products. Meat processing safety indicators include include total coliforms and Enterobacteriaceae [13].

In response to meat quality and safety concerns, numerous preservation methods have been developed, although no single approach provides comprehensive protection for all types of foods. Therefore, synthetic preservatives, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and class III butylated hydroquinone, have been routinely used to suppress microbial growth and extend the shelf life of meat [14]. However, these artificial preservatives have been linked to negative health outcomes such as disease and other illnesses. Because of their negative effects and perceived unsafety, natural alternatives have grown in popularity as consumers increasingly seek healthier products free of traditional chemical preservatives [15]. Rosemary extracts have been used due to their antioxidant, antifungal, and antimicrobial properties [16]. These properties are attributed to their chemical constituents such as carnosic acid [17]. Furthermore, the antioxidant activity of rosemary is mainly ascribed to its high content of isoprenoid quinones, which act as chain-breaking free radical scavengers and as chelators of reactive oxygen species [18]. These compounds also inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* [19].

Carnosic acid (CA) is a natural antimicrobial agent with stable chemical properties. Due to its antioxidant efficacy, it has been widely used as a food additive [20]. It also exhibits antibacterial activity against pathogens such as Staphylococcus aureus, *Escherichia coli*, and Salmonella [21]. Therefore, the bioactive compounds present in rosemary (Rosmarinus officinalis), particularly phenolic acids, contribute to reducing contamination levels in

meat by inhibiting the growth of harmful bacteria such as Escherichia E-coli [22].

Therefore, this study aimed to investigate the *in vitro* protective effect of purified carnosic acid against *Escherichia coli* and *Staphylococcus aureus* in experimentally treated broiler breast and thigh meat. The specific objectives were to: (1) assess the antimicrobial efficacy of three different concentrations of CA (50, 100, and 150 ppm) compared to an untreated control, and (2) evaluate the progression of microbial counts over storage periods of 3, 7, and 14 days at 4 °C. The findings of this research are intended to contribute scientifically grounded data on the potential of carnosic acid as a natural, effective intervention to enhance the microbiological safety and extend the shelf-life of broiler meat.

2. Materials and Methods

2.1. Sample Collection and Preparation

Broiler chickens (Gallus gallus domesticus) were obtained from a commercial farm in Basrah Governorate, Iraq, and humanely slaughtered at the farm's abattoir following standard procedures. Immediately post-slaughter, samples of pectoralis major (breast) and biceps femoris (thigh) muscles were aseptically collected. The external skin and visible adipose tissue were carefully removed using sterile instruments under laminar airflow conditions to minimize exogenous contamination. The samples were individually placed in pre-sterilized polyethylene bags (Whirl-Pak®, Nasco, USA), sealed, and immediately stored in insulated cooling containers with ice packs to maintain a temperature range of 2–4 °C during transport. The total sample weight was 4 kg, comprising 2 kg of breast meat and 2 kg of thigh meat. All samples were transported to the Public Health Laboratory at the College of Veterinary Medicine, University of Basrah, within one-hour post-slaughter. Upon arrival, under aseptic conditions in a biosafety cabinet, the meat samples were cut into homogeneous pieces of approximately 10 ± 2 g using sterile scalpels and cutting boards to ensure uniformity for subsequent treatments.

2.2. Experimental Treatment with Carnosic Acid

The phytochemical agent used was Carnosic Acid (CA), procured from Huamo Biotechnology Co. Ltd (Yongzhou, China; Product Code: HM-CA98, Batch No.: CA20231005). The compound had a certified purity of ≥98% (as per manufacturer's HPLC analysis report) and a stated validity until 2028.

A stock solution of CA was prepared by dissolving the compound in absolute ethanol (analytical grade, Sigma-Aldrich) to facilitate uniform application. The meat samples were randomly divided into four treatment groups for each meat type (breast and thigh):

Group 1 (Control): Treated with an equivalent volume of the ethanol vehicle only.

Group 2 (CA50): Treated with CA at a concentration of 0.05 g/kg meat (equivalent to 50 ppm).

Group 3 (CA100): Treated with CA at a concentration of 0.10 g/kg meat (equivalent to 100 ppm).

Group 4 (CA150):** Treated with CA at a concentration of 0.15 g/kg meat (equivalent to 150 ppm).

The CA solution (or ethanol for control) was applied drop-wise onto the surface of each meat piece using a sterile micropipette and then manually massaged gently with sterile gloves to ensure even distribution and complete coverage. After treatment, samples were placed back into sterile, labeled polyethylene bags, vacuum-sealed, and stored under refrigeration at 4 ± 1 °C. Microbiological analyses were performed

at predetermined storage intervals: 3, 7, and 14 days post-treatment.

2.3. Microbiological Analysis

At each sampling interval (Day 3, 7, and 14), 10 g of meat from each treatment group was aseptically weighed and homogenized with 90 mL of sterile 0.1% peptone water (Merck, Germany) in a stomacher (BagMixer®, Interscience, France) for 2 minutes to prepare a 10^{-1} dilution. Serial decimal dilutions were then prepared up to 10^{-6}.

Total Aerobic Bacterial Count (TBC): Enumeration of total aerobic mesophilic bacteria was performed by the standard pour plate method. One milliliter of appropriate dilutions was plated in duplicate on Plate Count Agar (PCA, HiMedia, India). Plates were incubated aerobically at 37 °C for 48 ± 2 hours. Results were expressed as Log_{10} colony-forming units per gram of meat (Log_{10} CFU/g).

Enumeration of *Escherichia coli*: Selective enumeration of E. coli was conducted using the spread plate technique on Eosin Methylene Blue (EMB) Agar (HiMedia, India). Duplicate plates of suitable dilutions were incubated at 44 °C for 24 hours. Characteristic metallic sheen colonies were counted and confirmed as E. coli by standard biochemical tests (Indole, Methyl Red, Voges-Proskauer, Citrate - IMViC). Counts were expressed as Log_{10} CFU/g.

Enumeration of *Staphylococcus aureus*: For the quantification of S. aureus, the spread plate method was employed on Baird-Parker Agar (HiMedia, India) supplemented with Egg Yolk Tellurite Emulsion. Plates were incubated at 37 °C for 24-48 hours. Presumptive S. aureus colonies (black, shiny, surrounded by a clear zone) were subjected to coagulase test (using rabbit plasma) for confirmation. Counts were expressed as Log_{10} CFU/g.

2.4. Statistical Analysis

All experiments were performed in triplicate (n=3). Microbiological data (Log_{10} CFU/g) were analyzed using one-way and two-way Analysis of Variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons, with a significance level set at $p < 0.05$. Statistical analyses were conducted using SPSS software (Version 26, IBM Corp., USA). Data are presented as mean ± standard deviation (SD).

3. Results

The results of the microbiological evaluation of broiler breast and thigh meat treated with varying concentrations of carnosic acid (CA) and stored at 4 °C for up to 14 days are presented in Tables 1 and 2. Bacterial counts, expressed as Log_{10} CFU/g, were derived from plate counts and analyzed to assess the antimicrobial efficacy of CA against total aerobic bacteria, *Escherichia coli*, and *Staphylococcus aureus*.

3.1. Effect on Total Aerobic Bacterial Count (TBC)

In both breast and thigh meat, the control groups exhibited a significant ($p < 0.05$) progressive increase in TBC over the 14-day storage period, reaching 5.99 Log_{10} CFU/g (corresponding to 9.70×10^5 CFU/g) and $9.27 \pm 0.32 \times 10^5$ CFU/g (5.97 Log_{10} CFU/g), respectively, by day 14 (Tables 1 & 2). In contrast, treatment with CA resulted in a significant ($p < 0.05$), dose-dependent suppression of total bacterial growth at all time points. For breast meat (Table 1), the lowest concentration (50 ppm) reduced TBC by approximately 1.3-1.4 Log_{10} cycles compared to the control across all storage days. The intermediate concentration (100 ppm) caused a reduction of 1.4-1.6 Log cycles, while the highest

concentration (150 ppm) demonstrated the most potent effect, achieving reductions of 1.7-1.9 Log cycles. A similar dose-responsive trend was observed in thigh meat (Table 2), with CA at 150 ppm consistently maintaining the lowest TBC throughout storage.

3.2. Effect on *Escherichia coli* Counts

The population dynamics of *E. coli* mirrored the overall TBC trend. Control samples showed significant growth, with counts in breast meat increasing from $6.33 \pm 0.14 \times 10^5$ CFU/g (5.80 Log_{10}) on day 3 to $8.76 \pm 0.06 \times 10^5$ CFU/g (5.94 Log_{10}) on day 14 (Table 1). CA treatment significantly ($p < 0.05$) inhibited *E. coli* in a concentration-dependent manner. Notably, the efficacy of CA against *E. coli* increased with storage time at higher concentrations. In breast meat treated with 150 ppm CA, *E. coli* counts were reduced by 1.3 Log cycles on day 3 and by a more substantial 2.8 Log cycles by day 14 compared to the respective controls. Thigh meat (Table 2) displayed a comparable pattern, where the 150-ppm treatment yielded the most significant suppression, particularly at the later storage intervals.

3.3. Effect on *Staphylococcus aureus* Counts

S. aureus was similarly susceptible to CA treatment. Control samples sustained high counts, exceeding 8.56×10^5 CFU/g (5.93 Log_{10}) in breast meat by day 14 (Table 1). All CA treatments led to significant ($p < 0.05$) reductions. The antimicrobial effect was markedly pronounced at higher concentrations. For instance, in breast meat, the 150-ppm treatment reduced *S. aureus* counts by 1.3, 1.7, and 2.1 Log cycles on days 3, 7, and 14, respectively, compared to the control. This progressive increase in efficacy over time was also evident in thigh meat samples (Table 2). Supporting these quantitative data, qualitative analysis on CHROMagar™ *S. aureus* revealed a stark reduction in the number of characteristic golden-colored colonies on plates inoculated with CA-treated meat homogenates, with near-complete absence observed at the 150-ppm concentration.

3.4. Molecular Analysis of *Staphylococcus aureus*

The antimicrobial effect of CA extended beyond culturability, as evidenced by molecular analysis. Polymerase Chain Reaction (PCR) targeting the species-specific *nuc* gene (756 bp) of *S. aureus* showed distinct, high-intensity amplicon bands in samples from the control group (Figure 2). In contrast, samples treated with CA, particularly at 100 ppm and 150 ppm, displayed a notable reduction in band intensity or a complete absence of the *nuc* amplicon. This suggests that CA treatment may impair bacterial DNA integrity or reduce the target bacterial load below the detection limit of the PCR assay.

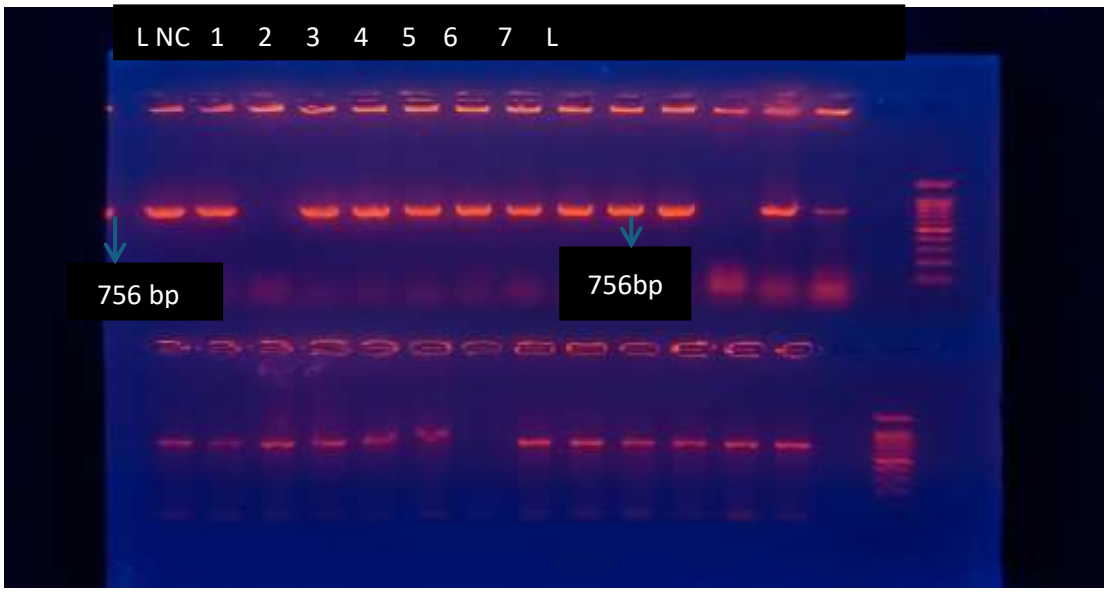

Figure 1. Agarose gel electrophoresis (1.5%) showing PCR amplification of the *nuc* gene (756 bp) from *Staphylococcus aureus* recovered from broiler meat samples. Lanes: M, 100 bp DNA ladder; C+, positive control (pure *S. aureus* culture); C-, negative control (no template); Ctrl, sample from untreated control meat; T2, T3, T4, samples treated with 50, 100, and 150 ppm carnosic acid, respectively. The diminishing band intensity correlates with increasing CA concentration.

In summary, the results demonstrate that carnosic acid possesses significant, dose-dependent antimicrobial activity against total aerobic bacteria, *E. coli*, and *S. aureus* in refrigerated broiler meat. Its efficacy increased over storage time and was corroborated by both traditional cultural methods and molecular detection techniques.

Table (1): Effect of adding different concentrations of carnosic acid on the Microbiology test of the breast chicken (x10^5).

Microbiology test	Time/ Treatment	3day	7day	14day
Total bacteria	Control	6.56 ±0.17 x10$^{5\ Ac}$	7.70 ±0.12 x10$^{5\ Ab}$	9.70±0.11 x10$^{5\ Aa}$
	0.05	4.60 ±0.11 x10$^{4\ Ba}$	2.70 ±0.11 x10$^{4\ Bb}$	4.46±0.23 x10$^{4\ Ba}$
	0.10	3.90 ±0.12 x10$^{4\ Ca}$	2.53 ±0.08 x10$^{4\ Bc}$	3.53±0.08 x10$^{4\ Cb}$
	0.15	3.30 ±0.11 x10$^{4\ Da}$	2.03 ±0.06 x10$^{4\ Cc}$	2.70±0.26 x10$^{4\ Db}$
	L. S. D.	0.434 *	0.322 *	0.622 *
E-Coli	Control	6.33 ±0.14 x10$^{5\ Ac}$	7.30 ±0.11 x10$^{5\ Ab}$	8.76±0.06 x10$^{5\ Aa}$
	0.05%	4.30 ±0.09 x10$^{4\ Ba}$	2.73 ±0.09 x10$^{4\ Bb}$	2.56±0.17 x10$^{4\ Bb}$
	0.10%	3.73 ±0.12 x10$^{4\ Ca}$	2.13 ±0.14 x10$^{4\ Cb}$	1.90±0.11 x10$^{4\ Cb}$
	0.15%	3.06 ±0.14 x10$^{4\ Da}$	1.70 ±0.11 x10$^{4\ Cb}$	1.30±0.12 x10$^{4\ Db}$

	L. S. D.	0.431 *	0.546 *	0.406 *
Staphylococcus aureus	Control	6.63 ±0.12 x10^{5} ^{Ac}	7.73 ±0.12 x10^{5} ^{Ab}	8.56±0.1 x10^{5}8 ^{Aa}
	0.05%	5.40 ±0.17 x10^{4} ^{Ba}	2.43 ±0.09 x10^{4} ^{Bb}	2.73±0.08 x10^{4} ^{Bb}
	0.10%	4.30 ±0.15 x10^{4} ^{Ca}	2.10 ±0.11 x10^{4} ^{Bb}	2.16±0.14 x10^{4} ^{Cb}
	0.15%	3.50 ±0.12 x10^{4} ^{Da}	1.60 ±0.12 x10^{4} ^{Cb}	1.46±0.15 x10^{4} ^{Db}
	L. S. D.	0.464 *	0.360 *	0.464 *

"Lowercase letters indicate significant differences between treatments within a column ($p < 0.05$). Uppercase letters indicate significant differences between storage days within a row ($p < 0.05$).

NS: mean Non-significant.

*: mean significant

Table (2): Effect of adding different concentrations of carnosic acid on the Microbiology test of the thigh chicken(x 10^{5})

Microbiology test	Time/ Treatment	3day	7day	14day
Total bacteria	Control	6.41±0.23 x10^{5} ^{Ab}	8.86±0.26 x10^{5} ^{Aa}	9.27±0.32 x10^{5} ^{Aa}
	0.05	4.11±0.02 x10^{4} ^{Bb}	4.70±0.11 x10^{4} ^{Ba}	4.56±0.17 x10^{4} ^{Ba}
	0.10	3.70±0.11 x10^{4} ^{BCa}	3.80±0.17 x10^{4} ^{Ca}	3.90±0.11 x10^{4} ^{Ca}
	0.15	3.30±0.11 x10^{4} ^{Ca}	3.03±0.08 x10^{4} ^{Da}	3.30±0.10 x10^{4} ^{Ca}
	L. S. D.	0.463 *	0.562 *	0.655 *
E-Coli	Control	6.62±0.18 x10^{5} ^{Ab}	6.59±1.72 x10^{5} ^{Ab}	9.70±0.30 x10^{5} ^{Ab}
	0.05	4.93±0.08 x10^{4} ^{Ba}	5.34±1.45 x10^{4} ^{Aa}	4.33±0.14 x10^{4} ^{Ba}
	0.10	4.23±0.17 x10^{4} ^{Ca}	3.10±0.17 x10^{4} ^{Bb}	3.43±0.17 x10^{4} ^{Cb}
	0.15	3.51±0.19 x10^{4} ^{Da}	2.23±0.08 x10^{4} ^{Bb}	2.56±0.18 x10^{4} ^{Db}
	L. S. D.	0.549 *	1.69 *	0.681 *

	Control	6.47±0.09 x10^{5} ^{Ab}	6.85±1.80 x10^{5} ^{Ab}	9.34±0.82 x10^{5} ^{Aa}
Staphylococcus aureus	0.05	5.30±0.30 x10^{4} ^{Ba}	5.75±1.70 x10^{4} ^{Aa}	5.03±0.29 x10^{4} ^{Ba}
	0.10	3.87±0.39 x10^{4} ^{Ca}	3.36±0.14 x10^{4} ^{Ba}	3.90±0.1 x10^{4} ^{BCa}
	0.15	3.76±0.08 x10^{4} ^{Ca}	2.50±0.11 x10^{4} ^{Bb}	2.96±0.29 x10^{4} ^{Cb}
	L. S. D.	0.835 *	2.06 *	1.512 *

"Lowercase letters indicate significant differences between treatments within a column ($p < 0.05$). Uppercase letters indicate significant differences between storage days within a row ($p < 0.05$).
NS: mean non-significant.
*: mean significant

4-Discussion

The results of this study showed that treating chicken meat with carnosic acid, especially at the higher concentrations (0.10g/kg) and (0.15g/kg), resulted in a significant decrease in the numbers of total bacteria, E. coli, and *Staphylococcus aureus* compared to the control treatment. An inhibitory effect on microbial growth was observed throughout the refrigerated storage period. These results are consistent with what [28], indicated, who explained that adding carnosic acid at a high concentration significantly reduces bacterial growth and microbial activity, and also limits the rise in chemical values indicative of meat spoilage, such as total volatile nitrogen and free fatty acids. this effect is due to the properties of phenolic compounds in rosemary, which act as antioxidants and antimicrobials, inhibiting bacterial proliferation and preventing the decomposition of proteins and fats during storage. The results of the current study also support the findings of other research, which demonstrated that the use of natural plant antioxidants significantly reduced the total bacterial count, helping to extend the shelf life of meat and preserve its sensory and physical properties [29,30]. This effect is attributed to the ability of phenolic compounds to penetrate bacterial cell walls and disrupt their vital components. Additionally, the accompanying phenolic compounds play a role in inhibiting oxidation and reducing moisture loss, which positively impacts meat quality [31]. Another study also demonstrated that a combination of natural phenolic acids possesses strong activity against antibiotic-resistant E. coli by damaging the cell membrane and reducing resistance proteins. This is consistent with our results, as the treatments showed a significant reduction in E. coli numbers compared to the control treatment. For the diagnosis of *Staphylococcus aureus*, CHROMagar Staph aureus medium demonstrated its high efficiency in detecting it with an accuracy exceeding 95%, making it a reliable and rapid diagnostic tool compared to conventional methods [32].

In addition [33], demonstrated that carnosic acids exhibited dual antioxidant and antibacterial activity, helping to reduce the number of harmful bacteria such as E. coli and staphylococci, while increasing the number of beneficial bacteria, and improving production performance and conversion efficiency. This enhances their potential as a natural alternative to antibiotics in poultry farming [33]. [34] Another study also demonstrated that the use of natural plant extracts improved the quality of chilled chicken meat and extended its shelf life. Carnosic acid was the most effective, preserving meat quality for up to 14 days thanks to its high content of phenolic compounds with antioxidant and antibacterial activity. Accordingly, the results of this study and previous studies confirm that the use of natural compounds such as carnosic acid represents a promising and effective approach to improving meat safety and extending its shelf life, reducing the need for artificial preservatives and enhancing the quality of food products.

5-Conclusion

Based on the investigation's findings, carnosic acid demonstrates significant potential as a natural antibacterial preservative for broiler meat. The study conclusively showed that treating chicken breast and thigh samples with varying concentrations of CA resulted in a

marked, concentration-dependent reduction in pathogenic bacteria, specifically *Escherichia coli* and *Staphylococcus aureus*, as well as the total bacterial count over a 14-day refrigerated storage period. By effectively inhibiting these key spoilage and pathogenic microorganisms, the application of carnosic acid presents a viable natural strategy to enhance the safety and extend the shelf life of highly perishable poultry products.

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