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RESEARCH ARTICLE
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EVALUATING THE INHIBITORY EFFECTS OF CITRIC ACID, GARLIC POWDER, AND OREGANO POWDER, ALONE AND IN COMBINATION, ON THE GROWTH OF *SALMONELLA ENTERITIDIS* STRAINS ISOLATED FROM CHICKEN MEAT DURING STORAGE

Ayesha kousar[1], Maria Khalid[2], Amina Zaib[3], Sofia Razzaq Meo[4], Maria Tariq[5], Azka Rizvi[6], Ali Ahmad sheikh[7*]

[1]University of Veterinary and Animal Sciences, Lahore Institute of Microbiology
[2]Senior Demonstrator at Services institute of medical sciences, Lahore, Pharmacology Department.
[3]University of Veterinary and Animal Sciences, Lahore Institute of Microbiology
[4]University of Veterinary and Animal Sciences, Lahore Institute of Microbiology
[5]University of Veterinary and Animal Sciences, Lahore Institute of Microbiology
[6]University of Veterinary and Animal Sciences, Lahore Institute of Microbiology
[7]*University of Veterinary and Animal Sciences, Lahore Institute of Microbiology

***Corresponding Author: -** Ali Ahmad sheikh
University of Veterinary and Animal Sciences, Lahore Institute of Microbiology,
Email: ali.ahmad@uvas.edu.pk

Abstract

The assurance of poultry meat quality and safety is a paramount concern for public health and trade. Pathogenic microorganisms, notably *Salmonella enteritidis*, jeopardize meat quality, necessitating effective interventions. This study investigated the antimicrobial efficacy of citric acid, garlic extract, and oregano seeds in reducing *Salmonella enteritidis* counts on chicken meat. Notably, *Salmonella enteritidis* is a leading foodborne pathogen associated with poultry products, contributing to substantial global gastroenteritis cases and fatalities. The emergence of drug-resistant strains due to indiscriminate antibiotic use underscores the urgency for viable alternatives. Spices such as garlic, oregano, and citric acid have gained attention for their potential antibacterial properties. In this research, comprehensive analyses were conducted on the effectiveness of these natural antimicrobial agents. Citric acid exhibited substantial inhibition zones at varying concentrations, leading to complete reductions in *Salmonella enteritidis* counts on chicken meat over different time intervals. Similarly, garlic extract demonstrated significant reductions in bacterial counts, with concentrations of 50%, 75%, and 100% exhibiting promising results. Oregano seeds' hexane extract also displayed potent antimicrobial properties, completely eradicating initial *Salmonella enteritidis* counts. Furthermore, the combined treatment of citric acid and garlic extract showcased synergistic antimicrobial activity. These findings underscore the potential of natural agents in enhancing food safety and quality by effectively reducing bacterial contamination in poultry products. The study contributes valuable insights into sustainable approaches for countering microbial threats in the food supply chain, ultimately ensuring consumer health and well-being.

Introduction

In an era of rising food safety and quality concerns, the preservation of perishable foods, particularly meat products, represents a significant challenge (Mc Carthy et al., 2018). *Salmonella enteritidis* stands out among the numerous pathogens that pose a threat to food safety due to its ability to cause severe foodborne illnesses (Campioni et al., 2012). Salmonellosis is a zoonotic problem and most prevalent source of Salmonellosis is poultry (CDC, 2008a). In 2007, 1.4 million cases of Salmonellosis accord in United States and productivity loss and medical cost was 2.5 billion dollars (USDA-ERS, 2007) (Hanning et al., 2009). To decrease the post slaughter contamination with pathogens, antimicrobials have been suggested (Mani-López et al., 2012). In the food industry, despite considerable progress taking into consideration the hygiene and control of meat consumption, however, Salmonella spp. is one of the most common food-related pathogen. In a summary report of the EU on the practice and sources of Zoonosis, Zoonotic agents and food outbreaks, the European Food Safety Agency (EFSA) says that reported 100,000 salmonellosis cases (per year) in the European Union. Among isolated serovars, the most common serovar was *Salmonella enteric* subspecies enteric serovar Enteritidis (*Salmonella enteritidis*), which accounted for 41.4% of reported cases in 2014, this number increased to 45.7% in 2015. they need to act as barriers and should be safe for human health(Stojanović-Radić et al., 2018). Frequently, improperly stored poultry, such as chicken meat, serves as a breeding ground for this pathogen, endangering consumer health (Collett et al., 2020). Antimicrobial agents have been used as food preservatives for centuries, with the incorporation of herbs, spices, and natural compounds known to inhibit microbial growth (Campêlo et al., 2019). Due to the development of antibiotic-resistant pathogen strains, organic acids and their salts are now recognised by European law as an alternative source of antibiotics in the diet. Citric acid is utilised as a natural preservative in weak organic acids because it has the most inhibitory impact as it diffuses through the cell membrane and causes the cytoplasm to become acidic (Firouzabadi et al., 2014). Due to their broad-spectrum antimicrobial properties and established safety, citric acid, garlic powder, and oregano powder have emerged as promising candidates (Lima et al., 2021). Citric acid, a weak organic acid, has been shown to disrupt microbial cell membranes and enzymes (Rattanaporn et al., 2018), thereby inhibiting bacterial proliferation. Garlic and oregano, both of which contain bioactive compounds such as allicin and thymol, exert antimicrobial activity by interfering with vital cellular processes (Redondo-Blanco et al., 2020). In Pakistan, the poultry industry plays a significant role in the food industry, and chicken products are used throughout the country to meet protein needs. However, the poultry industry suffers enormous economic losses due to bacterial infections. Salmonella is the most common cause of bacterial infections, and *Salmonella enteritidis* is the most common cause of non-typhoidal salmonellosis in humans. Annually, 94 million cases of gastroenteritis caused by Salmonella species are estimated to occur globally, resulting in 155,000 fatalities (Asif et al., 2017). Individual studies have investigated the inhibitory effects of these antimicrobial agents on a variety of pathogens, including Salmonella species. However, a comprehensive investigation of their combined activity and effect on *Salmonella enteritidis* growth on chicken meat during storage is still relatively unexplored. Understanding how these agents interact and whether their combination produces additive or synergistic effects could pave the way for more effective and safer food preservation strategies. This study aims to fill this void by systematically evaluating the antimicrobial potential of citric acid, garlic powder, and oregano powder, both individually and in combination, thereby contributing valuable insights to the fields of food science and safety.

Methodology

Sampling

Anhydrous citric acid, garlic cloves, oregano seeds and chicken breast meat was purchased from Lahore's local market chicken was stored at -20℃ in the Central Laboratory Complex, University of Veterinary and Animal Sciences, Pattoki.

Preparation of solutions

Citric acid solution was prepared in sterile water 1-5% (weight/volume), Garlic mesh prepared and considered as 100% pure, further diluted as 75%, 50% and 25%.
Oregano seeds extraction was performed by Hexane using Soxhlet apparatus at 70℃ for 4 hours (Rahbar et al., 2012).

Salmonella enteritidis

Glycerol preserved ATCC culture of *Salmonella enterica* subsp. *enterica* serovar *Enteritidis* (ATCC® 13076™) revived by sub-culturing into the Trypto Soya broth. Which was later inoculated on Salmonella Shigella agar (SS agar) for colony isolation.

Antimicrobial activity testing

1. Making bacterial cultures:

SS agar plates with a *Salmonella enteritidis* bacterial culture should be revived.
A bacterial colony was transferred from an agar plate into a 10 ml sterile Tryptic Soy Broth (TSB) tube.
The TSB tube than incubated at 37°C for 18 hours to promote logarithmic bacterial growth.

2. Petri plate preparation:

Preparation of 20 ml of Muller Hinton agar (MHA) for Petri plates.

3. Inoculation with bacteria:

Got a suspension of *Salmonella enteritidis* that was adjusted to a 0.5 MacFarland standard and has a concentration of 1.5 108 colony-forming units per millilitre (CFU/ml) (Optical Density 0.08–0.1).
Spread the Salmonella suspension evenly across the MHA surface in the Petri plates using a sterile cotton swab.

4. Agar Wells preparation:

Used a sterile pippete tip to create 6mm-diameter agar wells in the MHA plates using the sterilised tip.

5. Antimicrobial Agents Addition

Prepared various antimicrobial agent concentrations using a micropipette (Cleaver Scientific):
Prepared solutions of citric acid at 3%, 4%, and 5% concentrations.
Prepared solutions with concentrations of 50%, 75%, and 100% for garlic extracts
Hexane Oregano Seed Extract: Make a solution with a 100% concentration.
Each of the antimicrobial agents' concentrations must go into its corresponding well on the MHA plates with 100 l.

6. Incubation

Put the prepared Petri plates upright inside a 37°C-set incubator (such as the Galaxy 170S New Brunswick Incubator).

7. The Incubation Phase:

The plates were incubated for 24 hours in order to promote bacterial growth and possible inhibition.

8. Inhibition zone measurement

Checked the plates for the presence of inhibition zones around the wells after the incubation period. Used a ruler or other measuring tool, determine the diameter of the inhibition zones in millimetres.

Meat exposure with growth

One gramme of chicken meat was air dried after being sterilised in hot water (80°C) for 20 minutes. Using spectrophotometry, a *Salmonella enteritidis* inoculum (0.5 MacFarland) was created. To allow for bacterial attachment, the chicken pieces were exposed to the inoculum for 20 minutes. Chicken pieces that had not been treated or exposed to bacteria were included in the control groups.

Synergistic effect of Citric acid and garlic

Sub-therapeutic levels of citric acid and garlic extract were combined to explore synergistic effects. Combination Group 1 (C1) contained 50% garlic solution and 1% citric acid. Combination Group 2 (C2) comprised 0.75% citric acid and 75% garlic solution. Combination Group 3 (C3) included 0.05% citric acid and 100% garlic extract.

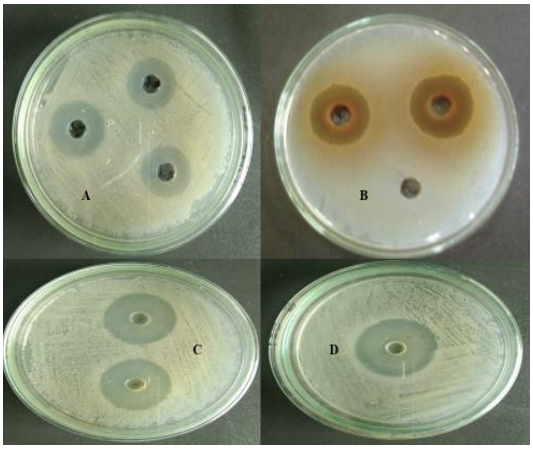
Statistical Design:

The data collected was gathered in spreadsheet (MS Excel 2016)**.** Statistical analysis was done to see the efficacy of various extracts on survival of *Salmonella enteritidis* on chicken meat using commercially available software of Statistical Package for Social Sciences (SPSS) was opted to process the obtained date through one-way analysis of variance (ANOVA). The *p*-value of 0.05 was considered significant

Results

Antimicrobial activity citric acid, garlic and oregano seeds

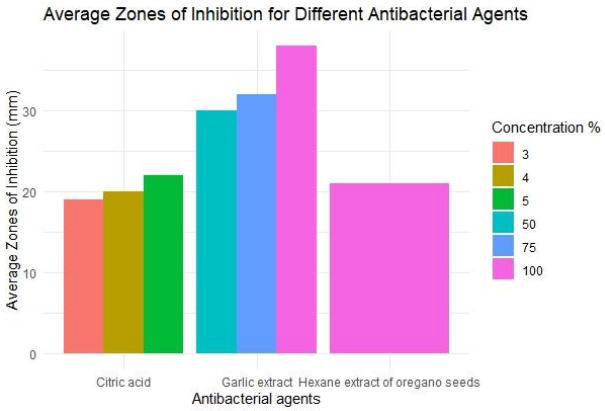
The antibacterial effects of garlic extract were assessed against *Salmonella enteritidis* on MH agar using well diffusion assay. Inhibition zones of 30 mm, 32 mm, and 38 mm were observed with 50%, 75%, and 100% garlic extract concentrations, respectively. Similarly, pure hexane extract of oregano seeds exhibited antibacterial activity, resulting in inhibition zones of 22 mm and 21 mm. However, hexane extracts of oregano leaves did not produce any growth inhibition. Furthermore, citric acid solutions at 3%, 4%, and 5% concentrations demonstrated antibacterial activity on MH agar against *Salmonella enteritidis*. Inhibition zones of 19 mm, 20 mm, and 22 mm were recorded for 3%, 4%, and 5% citric acid solutions, respectively. Shown in figure below



A; **Citric acid**, B; **Oregano seeds, C and D: garlic extract against *Salmonella enteritidis***

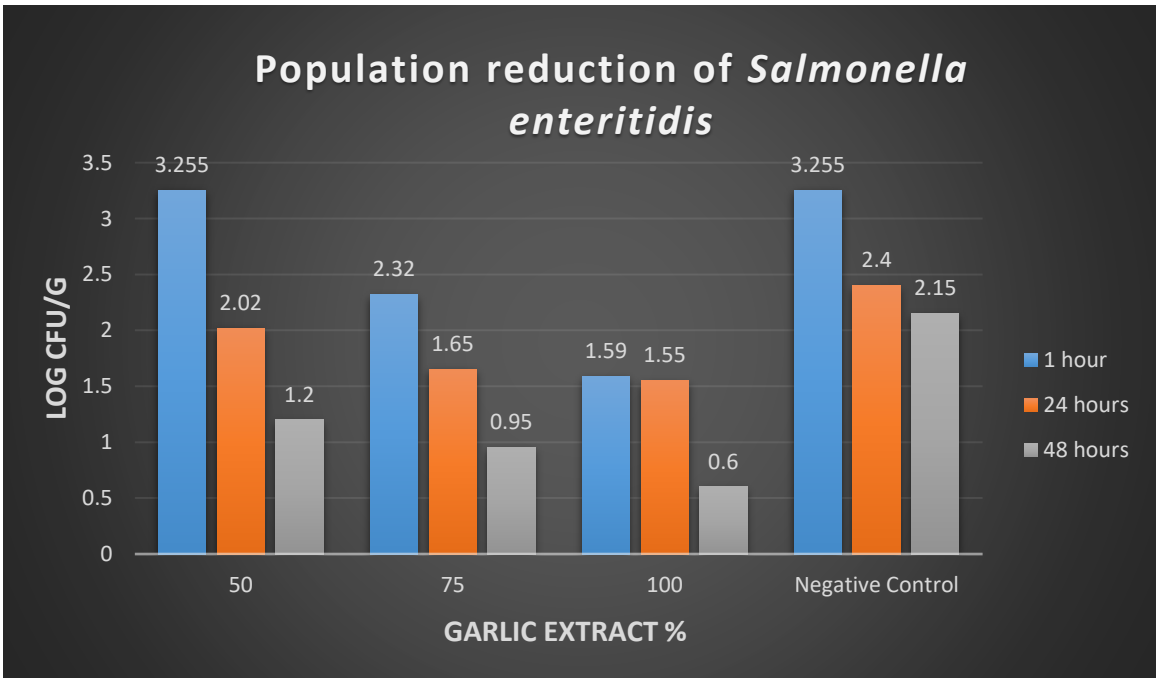
The graph presents data on the average zones of inhibition (mm) exhibited by various antibacterial agents at different concentrations. Antibacterial agents include Citric acid, Garlic extract, and Hexane

extract of oregano seeds. The concentrations vary for each agent, and the graph visually represents the relationship between antibacterial agents, concentration percentages, and zones of inhibition

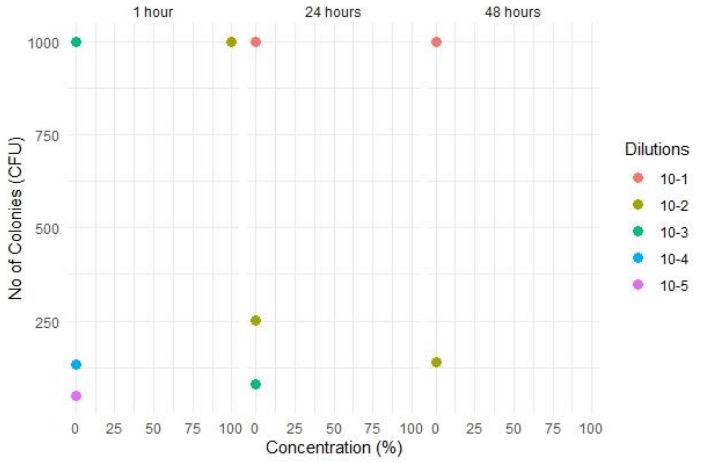


Citric acid antimicrobial activity against *Salmonella enteritidis* in comparison with Control
Treatment with citric acid solutions at concentrations of 2%, 3%, 4%, and 5% effectively eliminated initial *Salmonella enteritidis* counts (0.176 log CFU/g) on chicken meat within 1 to 48 hours at 4°C. In contrast, the untreated control group showed no reduction in bacterial count. Application of a 1% citric acid solution for one hour resulted in a partial reduction (0.11 log CFU/g) compared to the control, reaching complete elimination after 24 hours.

Efficacy of Garlic Extract on Reduction of *Salmonella enteritidis* in comparison with Control
A significant reduction in initial *Salmonella enteritidis* count (0.176 log CFU/g) to 0.11 log CFU/g through treatment with Garlic Extract solutions of 50%, 75%, and 100% over 1 and 24 hours at 4°C. This reduction was notably lower compared to the control group. Furthermore, complete elimination of *Salmonella enteritidis* on chicken meat was achieved after 48 hours. Conversely, the untreated control group exhibited no alteration in bacterial count, highlighting the effective antimicrobial impact of the Garlic Extract treatment.



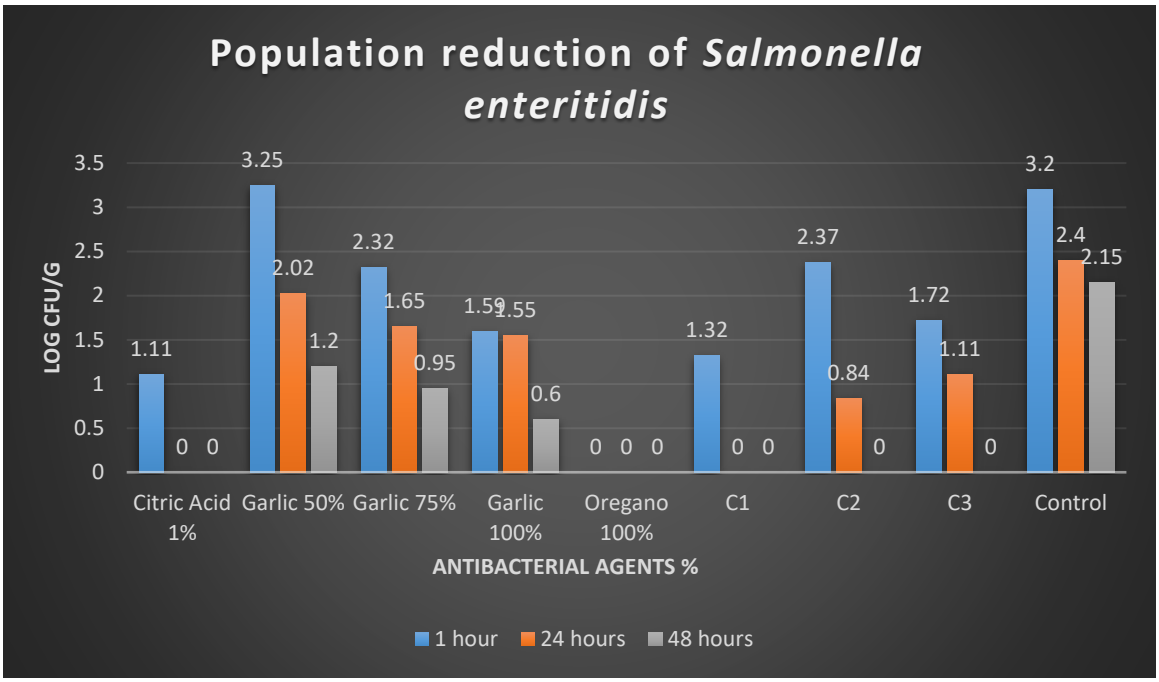
A complete reduction of initial *Salmonella enteritidis* count (0.176 log CFU/g) on chicken meat through Hexane Extract of Oregano Seeds (100%) treatment within 1, 24, and 48 hours at 4°C. Conversely, the control group exhibited unchanged bacterial count, emphasizing the effective antimicrobial action shown in figure



Efficacy of Citric acid and Garlic Extract in combinations on Reduction of *Salmonella enteritidis* in comparison with Control

Initial *Salmonella enteritidis* count (0.176 log CFU/g) decreased to 0.11 log CFU/g with C1, C2, and C3 combination solutions (Citric acid and Garlic Extract) after 1 and 24 hours at 4°C. Reduction persisted at 48 hours. Control group exhibited no change, reinforcing antimicrobial efficacy shown in table below

Treatment time	Concentration of combination solutions	Dilutions of Combination solutions	No of colonies (CFU) on SSA plates	Dilutions (Negative Control group)	No of (colonies negative Control group)
1 hour	**C1** (1% citric acid + 50% garlic extract)	10^{-1}	TNTC	10^{-1}	TNTC
		10^{-2}	21		
		10^{-3}	0		
		10^{-4}	0		
	C2 (0.75% citric acid + 75% garlic extract)	10^{-1}	TNTC	10^{-2}	329
		10^{-2}	239	10^{-3}	39
		10^{-3}	10		
		10^{-4}	0		
	C3 (0.5% citric acid + 100% garlic extract)	10^{-1}	TNTC		
		10^{-2}	53		
		10^{-3}	20	10^{-5}	0
		10^{-4}	0		
24 hours	**C1** (1% citric acid + 50% garlic extract)	10^{-1}	3	10^{-1}	TNTC
		10^{-2}	1		
		10^{-3}	0		
	C2 (0.75% citric acid + 75% garlic extract)	10^{-1}	47	10^{-2}	228
		10^{-2}	7		
		10^{-3}	0		
	C3 (0.5% citric acid + 100% garlic extract)	10^{-1}	136	10^{-3}	11
		10^{-2}	13		
		10^{-3}	0		
48 hours	**C1**	10^{-1}	0	10^{-1}	TNTC
	C2	10^{-1}	0		
	C3	10^{-1}	0		
		10^{-2}	0		



*C1(1% Citric acid+50% Garlic Extract) *C2(0.75% Citric acid+75% Garlic Extract *C3(0.5%Citric acid+100%Garlic Extract * Control=Untreated bacterial exposed group

Comparison of effect of antibacterial agents' Citric acid, Garlic Extract and Hexane Extract of Oregano seeds on *Salmonella enteritidis* onto poultry meat, with comparison to negative control group (untreated, bacterial exposed group).

Statistical analyses revealed noteworthy findings across various treatments. Citric acid exhibited substantial reductions in *Salmonella enteritidis* count at concentrations of 1%, 2%, 3%, 4%, and 5%, as well as significant differences when compared to controls. Additionally, differing concentrations displayed significant variations among themselves. Garlic extract, at 50%, 75%, and 100%, induced notable reductions compared to controls, while concentrations presented no significant differences. Application of Citric acid and Garlic Extract combinations (C1, C2, and C3) led to substantial reductions versus controls. Temporal variations were also statistically significant for these treatments as given in table below

Treatment	Concentration/Combination	Significant Reduction	P-value	Time Intervals Significant
Citric Acid	1%, 2%, 3%, 4%, 5%	Yes	<0.05	Yes
Citric Acid	2%, 3%, 4%	Yes	<0.05	Yes
Garlic Extract	50%, 75%, 100%	Yes	<0.05	No
Citric Acid + Garlic Extract	C1, C2, C3	Yes	<0.05	Yes

Discussion

The preservation of poultry meat quality and safety holds paramount significance in contemporary diets, ensuring public health and trade viability. Pathogenic microorganisms, including *Salmonella enteritidis*, pose threats to meat quality. Notably, *Salmonella enteritidis* is a significant foodborne pathogen linked to poultry products (Antunes et al., 2016), with its prevalence causing substantial global gastroenteritis cases and related fatalities (Jajere, 2019). An escalating challenge is the emergence of drug-resistant *Salmonella enteritidis* strains, attributed to unwarranted antibiotic usage in food and livestock domains (Hoelzer et al., 2018). These strains can traverse from poultry to humans, necessitating robust mitigation strategies (MacKenzie et al., 2017). For poultry processors and food safety researchers, effective bacterial load reduction in meat is imperative. Spices like garlic,

oregano, and citric acid have been acclaimed for their antibacterial properties (Xu et al., 2021). The study outcomes demonstrated the efficacy of citric acid in generating zones of inhibition with diameters of 19 mm, 20 mm, and 22 mm at concentrations of 3%, 4%, and 5%, respectively. Notably, treatment with citric acid solutions of 2%, 3%, 4%, and 5% at 1, 24, and 48 hours at 4°C led to the complete reduction of the initial *Salmonella enteritidis* count (0.176 log CFU/g) on chicken meat, while no change occurred in the control group. Treatment with a 1% citric acid solution for one hour reduced the count to 0.11 log CFU/g, comparatively lower than the control group, with complete reduction achieved after 24 hours. Aqueous citric acid solutions exhibited significant antibacterial efficacy, leading to the increased vulnerability of bacterial membranes and swift pathogen inactivation (Dudeja et al., 2023). In the context of garlic extracts, inhibition zones of 30 mm, 32 mm, and 38 mm were achieved at concentrations of 50%, 75%, and 100%, respectively. Treatment with 50%, 75%, and 100% Garlic Extract solutions for 1 and 24 hours at 4°C led to a notable reduction of *Salmonella enteritidis* count from 0.176 log CFU/g to 0.11 log CFU/g, exceeding the control group. Further, complete elimination was observed after 48 hours, while the control group remained unchanged. The antimicrobial potency of garlic, an ancient remedy, is well documented in a study The mixture of honey and A. sativum has a synergistic antimicrobial effect on all strains tested, according to the results. Additionally, compared to honey and their combination, A. sativum demonstrated higher antioxidant activity as well as higher TPC and TFC (Abderrahim et al., 2019). However, its efficacy is subject to time, temperature, pH, and other influencing factors, as evidenced by the gradual decline of *Salmonella enteritidis* count on chicken meat (Noori et al., 2018). Hexane extract of oregano seeds demonstrated inhibition zones of 21 mm and 22 mm. Furthermore, treatment with pure hexane extract of oregano seeds led to the complete eradication of the initial *Salmonella enteritidis* count (0.176 log CFU/g) on chicken meat after 1, 24, and 48 hours at 4°C, without any change in the control group. Oregano, an established natural product, offers multifaceted health benefits, including antibacterial, antioxidant, anticoccidial, and antifungal attributes (Ali et al., 2021). The combined treatment of citric acid and garlic extract (C1, C2, and C3) exhibited remarkable outcomes, reducing the initial *Salmonella enteritidis* count to zero. This effect was observed after 48 hours at 4°C for solutions C1 (1% citric acid + 50% garlic extract), C2 (0.75% citric acid + 75% garlic extract), and C3 (0.5% citric acid + 100% garlic extract), presenting significant contrast to the control group. In all scenarios, the untreated control group showed no alteration in bacterial count. Intestinal flora and productivity can be improved by including dietary supplements and their combinations, especially the probiotic and citric acid combination (Elbaz et al., 2021). This study underscores the potential of citric acid, garlic extract, and oregano seeds to effectively reduce *Salmonella enteritidis* counts on chicken meat, offering promising strategies for enhancing food safety and quality. These findings provide valuable insights into the utilization of natural antimicrobial agents to counteract bacterial contamination in poultry products, ultimately safeguarding consumer health and welfare.

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