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Garlic Juice as a Food Additive to Control Microbial Contamination in Poultry Meat

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ABSTRACT

Background: This study aims to evaluate the antibacterial activity of garlic extracts in fresh poultry meat. **Methods:** The antibacterial properties of garlic extracts were tested against *Salmonella typhimurium*, *Bacillus cereus*, *Escherichia coli*, *Micrococcus luteus*, and *Proteus mirabilis*. Antibacterial activity was assessed using disc and well diffusion methods. The minimum inhibitory concentration (MIC) was determined via macrodilution. Moreover, ground poultry meat samples were treated with garlic juice and cold macerate natural antibacterial preservatives at their minimum bactericidal concentrations (MBCs) and stored at 4 °C for 24 hours. **Results:** Garlic juice exhibited stronger antibacterial effects compared to the macerate. The highest inhibition zone was observed against *Micrococcus luteus* (23.33±1.11 mm). The MICs for garlic juice and cold macerate were 250 and 300 µl/ml, respectively. Furthermore, MBC values ranged from 250 to 300 µl/ml for garlic juice and 300 to 500 µl/ml for the macerate. The addition of garlic juice significantly reduced bacterial counts in refrigerated poultry meat compared to untreated controls. **Conclusion:** The findings suggest that garlic juice possesses potent antimicrobial properties and can enhance the microbial safety and preservation of poultry meat during cold storage.

Introduction

Poultry meat is an excellent source of essential amino acids with high biological value, unsaturated fatty acids, and minerals, while also being characterized by low lipid content and relatively low production costs (Noori *et al.*, 2018). However, its high pH and moisture content make it particularly susceptible to lipid and protein oxidation, as well as microbial growth under aerobic conditions, which significantly reduces its shelf life (Latou *et al.*, 2014, Lorenzo *et al.*, 2014). Furthermore, chicken meat is highly perishable due to contamination by pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli*, *Campylobacter jejuni* and *Salmonella* spp. (Woraprayote *et al.*, 2016).

Foodborne diseases, in addition, have a substantial economic impact on both the food industry and public health systems, primarily due

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to productivity losses, medical treatment costs, and the burden of food safety management (Lorenzo *et al.*, 2018). These diseases are also responsible for nearly 600 million illnesses and approximately 420,000 deaths worldwide each year (Mexis *et al.*, 2012).

Currently, a major challenge in the meat industry is to enhance the stability, shelf life, and overall acceptability of chicken meat by delaying lipid oxidation and inhibiting microbial growth. Concerns over the potential health risks associated with synthetic preservatives such as sodium nitrate, benzoic acid, and potassium sorbate have prompted researchers and the meat industry to explore the use of natural alternatives. These include bacteriocins (Lorenzo *et al.*, 2018), organic acids (Mexis *et al.*, 2012), essential oils (EOs) (Simona *et al.*, 2021), and chitosan (Latou *et al.*, 2014), which have demonstrated effectiveness in delaying lipid and protein oxidation.

In recent years, considerable research has focused on investigating the antibacterial activity of natural products (Bal *et al.*, 2019, Mohammed *et al.*, 2020). Among these, plants, particularly herbs and spices, have garnered growing attention due to their bioactive compounds. Currently, more than one thousand plant species are known to possess antimicrobial properties, and over 30,000 antimicrobial substances have been isolated from plants (Mohammed *et al.*, 2019, **Sevindik**, **2018, Zheng** *et al.*, **2016**). Spices have been used for centuries not only as food and flavoring agents but also, more recently, as natural remedies and food preservatives. Numerous spices, including cinnamon, clove, turmeric, oregano, rosemary, and thyme, have been shown to protect food from contamination by pathogenic bacteria (Jessica Elizabeth *et al.*, 2017, Sevindik, 2018).

Garlic is well known for its diverse phytochemical properties. Its bioactive molecules play a pivotal role in supporting human physiological functions and have the potential to prevent or alleviate various health conditions. The global consumption of traditional medicinal plants, particularly garlic, has increased steadily due to their high efficacy, minimal side effects, and relatively low cost (Venkatesh *et al.*, 2003). Scientific studies have shown that garlic consists of approximately 65% water, 30% carbohydrates, and 5% other bioactive components, mainly sulphur- and non-sulphur-containing compounds. Among these, organosulfur compounds such as cysteine sulphides and thiosulfates are of particular importance (Tapiero *et al.*, 2004).

In the present study, the authors investigated the in vitro antimicrobial activity of various garlic extracts and conducted a microbiological assessment of the sanitary quality of chicken meat, which is commonly produced and sold by local butchers. Finally, the authors evaluated the preservative effects of garlic extracts on chicken meat during refrigerated storage.

Materials and Methods

Plant source and identification

The test plant, *Allium sativum* L. (garlic), was purchased from a local vegetable market in Bordj Bou Arreridj, Algeria.

Strains and culturing conditions

Five bacterial strains were used in this study: *Escherichia coli* ATCC 25922, *Proteus mirabilis* ATCC 35659, *Salmonella typhimurium* ATCC 13311, *Bacillus cereus* ATCC 10876, and *Micrococcus luteus* ATCC 10240. The bacterial cultures were stored at –20 °C in nutrient broth supplemented with 20% (v/v) glycerol. Prior to each experiment, approximately 20 µl aliquots of the frozen cultures were transferred into tubes containing 5 ml of nutrient broth and incubated overnight at 37 °C.

Preparing the garlic extract

Preparation of garlic essential oil (GEO): A Clevenger-type apparatus was used for the extraction of GEO. Fresh garlic material (400 g) was immersed in 1000 ml of water and subjected to hydro-distillation. The obtained essential oil was collected, stored in a dark glass bottle, and kept at 4 °C until further use. Finally, the extraction yield of garlic essential oil was calculated.

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Hot maceration (HM): One hundred grams of crushed garlic were mixed with 500 ml of water in a 1-liter flask. The mixture was macerated for 1 hour starting from the boiling point. After maceration, the solution was filtered to remove solid residues. The pH of the macerate was measured, and the macerate was stored at 4 °C until further use.

Cold maceration (CM): One hundred grams of crushed garlic were mixed with 500 ml of water in a 1-liter flask. The mixture was macerated for 24 hours at room temperature (approximately 20 °C). A stopper was placed on the flask to prevent the loss of volatile compounds. After maceration, the solution was filtered to remove solid residues. Then, the pH of the macerate was measured, and the macerate was stored at 4 °C until further use.

Garlic juice (GJ): Five hundred grams of garlic bulbs were cleaned and peeled to remove all outer layers. The cloves were weighed before being placed in a blender (Silver Crest brand). Fresh garlic juice was obtained by grinding and filtering through a gauze cloth. The filtrate was then centrifuged at 3000 rpm for 20 minutes, and the supernatant was collected. The juice was stored at 4°C in a sterile, hermetically sealed glass bottle to prevent the loss of volatile compounds and wrapped in aluminum foil (Benmeddour *et al.*, 2015).

Sterility of the extracts: Sterility of extracts, i.e., the presence or absence of living microorganisms, is a critical parameter to be adhered to in order to ensure safety as well as quality of the final product. Sterility was maintained by filtering the extracts through 0.22-µm pore-size membrane filters. Thereafter, 1 ml of each sterile extract was aseptically transferred into individual test tubes containing 5 mL each of sterile nutrient broth. The test tubes were incubated at 37 °C for 24 hours. Following the incubation period, there were no signs of turbidity on all samples, which signifies the absence of microbial growth and the sterility of the extracts (Anyamaobi *et al.*, 2020).

Evaluation of in vitro antibacterial activity of garlic extracts

The antibacterial activity of garlic extracts was evaluated using the agar disc diffusion method as described by Balouiri (Balouiri *et al.*, 2016). Mueller-Hinton agar plates were inoculated by spreading 0.1 ml of an overnight bacterial culture adjusted to 0.5 McFarland turbidity standards on the surface. Whatman No. 3 absorbent disks (6 mm diameter) were impregnated with 20 µl of each extract and placed on the inoculated agar plates (90 mm diameter). Also, disks impregnated with distilled water served as negative controls, while Clamoxyl antibiotic disks were used as positive controls for comparison. The plates were left at room temperature for one hour to allow proper diffusion of the extracts before incubation at 37 °C for 24 hours. The diameters of the inhibition zones were measured in millimeters and recorded. All tests were performed in triplicate. Sensitivity was interpreted according to Ponce *et al.* as follows: resistant (< 8 mm), sensitive (9–14 mm), and very sensitive (15–19 mm) (Ponce *et al.*, 2003).

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC)

The MIC was determined by identifying the lowest concentration of the extract that inhibited visible growth of the test organisms, following the method described by Doughari (Doughari *et al.*, 2007) . In each test tube, 2 ml of nutrient broth was mixed with 0.5 ml of garlic extract at varying concentrations (v/v with distilled water): 500 µl/ml, 300 µl/ml, 250 µl/ml, 175 µl/ml, 62 µl/ml, and 3 µl/ml. A loopful of each bacterial suspension, previously adjusted to 0.5 McFarland turbidity standards, was then added to each tube. The same procedure was applied to all test organisms across the range of extract concentrations. A test tube containing only nutrient broth and a loopful of bacterial suspension served as the growth control. All test tubes were incubated at 37 °C for 24 hours, after which they were

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examined for turbidity. The lowest concentration of the extract at which no visible turbidity was observed was recorded as the MIC. To determine the MBC, a loopful of inoculum from each broth tube was subcultured onto solid agar plates divided into six sections. The lowest concentration of garlic juice (GJ) and cold macerate (CM) that resulted in <0.1% survival of the original bacterial inoculum was recorded as the MBC, according to the criteria described by Forbes (Forbes *et al.*, 1998). The antibacterial potency of each extract was further assessed by calculating the MBC/MIC ratio.

Microbiological analysis of poultry meat

In the laboratory, the meat sample was divided into five units and cut into small pieces using a sterile Petri dish. Each unit was weighed precisely (25 g) in a pre-weighed Stomacher bag. Then, 225 mL of tryptone salt solution (TSE) (Pasteur Institute, Algeria) was added to each bag. The samples were homogenized using a Stomacher for 2–3 minutes. The resulting homogenate (stock solution, SM; 10^{-1} dilution) was transferred into sterile bottles and left to stand for 45 minutes to allow reactivation of shocked or stressed microorganisms. Serial ten-fold dilutions were then prepared from the stock solution using tryptone salt solution (TSE; Pasteur Institute, Algeria), following the guidelines of ISO 6887-1 (1999). The microbial groups enumerated included total viable aerobic bacteria, total coliforms, and fecal coliforms.

The total viable aerobic bacteria were evaluated according to ISO 4833 (2003) using the aerobic plate count (APC) method. Samples were plated on Plate Count Agar (PCA) and incubated at 30 °C for 24–48 hours. Total coliforms were determined using the Most Probable Number (MPN) technique with Brilliant Green Bile Lactose Broth (BGB). Fecal coliforms were assessed using the same MPN method, with incubation at 44.5 °C for 48 hours, in accordance with ISO 7251 (ISO SR. 7251, 2005).

All microbial counts were expressed as the decimal logarithm of colony-forming units per gram ($\log_{10}$ CFU/g). The results were compared with the microbiological quality criteria established by the (Interministerial decree of 4th octobre, 2016)(Interministerial decree of 4th octobre, 2016)(Interministerial decree of 4th octobre, 2016)Algerian Interministerial Decree of July 2, 2017, concerning food safety standards. According to this decree, the maximum acceptable limits are as follows: Total aerobic bacteria: 5×10^5CFU/g, total coliforms: 5×10^3 CFU/g , fecal coliforms: 5×10^2 CFU/g (Interministerial decree of 4th octobre, 2016).

Antimicrobial activity of garlic extracts in poultry meat

The raw material (chicken meat, weighing between 2.5–5 kg) was purchased from a local butcher and immediately transported to the laboratory in ice boxes. Chicken breast meat was randomly divided into three experimental groups as follows: T1 (Negative Control): No treatment applied, T2: Garlic juice at its minimum bactericidal concentration (MBC; 300 µl/ml) added per gram of breast meat; T3: Cold garlic macerate at its MBC (300 µl/ml) added per gram of breast meat. All samples were stored under refrigeration (4 °C) for a total of 16 days. Microbial counts, including total viable aerobic bacteria, total coliforms, and fecal coliforms, were performed at four-day intervals. At the end of the storage period, the percentage of microbial inhibition was calculated.

$$\%\ of inhibition = \frac{log 10\ CFU/g\ (CM) - log\ 10\ CFU/g\ (TM)}{log\ 10\ CFU\ \ (CM)}\ X\ 100$$

log_{10}CFU/g: Decimal logarithm of colony-forming units per gram. CM: Control meat; TM: Treated meat.

Data analysis

Results were expressed as mean±standard deviation. The differences between groups were determined by analysis of variance (one-way ANOVA) using SPSS software (Statistical Product and Service Solutions versions 21.0, 2011). Differences were considered significant at P-value< 0.05.

Results

Antimicrobial activity by agar diffusion method

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results

The antibacterial activity of garlic extracts against the various bacterial strains is presented in **Table 1**. The observations reveal that garlic juice exhibited the highest antimicrobial efficacy among all tested extracts. It demonstrated superior inhibitory activity compared to both the essential oil and cold macerate extract across all bacterial strains, with the largest zone of inhibition measuring 23.33 mm against *Micrococcus luteus*. In contrast, the essential oil and cold macerate extract produced maximum inhibition zones of 19.83 mm and 19.33 mm, respectively, against the same strain. Notably, the hot macerate extract did not exhibit any observable antibacterial activity, indicating a loss of efficacy due to heat treatment. Furthermore, the inhibition zone of garlic juice against *Bacillus cereus* was statistically comparable to that produced by the standard antibiotic Clamoxyl. Additionally, inhibition zones for *Salmonella typhimurium* and *Proteus mirabilis* were significantly larger when treated with garlic juice compared to Clamoxyl, suggesting enhanced antibacterial potency. In terms of susceptibility, all tested strains demonstrated 100% sensitivity to garlic juice. Conversely, a high prevalence of resistance was observed with the heat-treated macerate and garlic essential oil, with resistance rates of 80% and 60%, respectively, as illustrated in **Figure 1**.

Table 1. Inhibition zone diameters (in mm) of different extracts.

Garlic extract	Strains				
	E. coli	***S. typhimurum***	***P. mirabilis***	***M. luteus***	***B. cereus***
GEO	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{b}	19.83 ± 0.77^{b}	29±3.33*
CM	9.66 ± 0.44^{b}	9.5 ± 1.0^{b}	9.16 ± 1.22^{c}	19.33 ± 0.55^{c}	11.66 ± 0.44^{ns}
HM	8.83 ± 0.22^{b}	2.66 ± 0.55^{c}	7.83 ± 0.22^{c}	9.00 ± 0.66^{c}	7.66 ± 0.55^{ns}
GJ	15.33 ± 0.44^{c}	20.16 ± 0.55^{b}	19.66 ± 0.44^{c}	23.33 ± 1.11^{c}	15.66 ± 0.77^{ns}
Clm	23.5±0.0	18.0±0.0	17.0±0.0	26.0±0.0	15.5±0.0

GEO: Garlic essential oil, **CM**: Cold macerate, **HM:** Hot macerate, **GJ**: Garlic juice, and **Clm**: Clamoxyl. Values expressed as mean ± SD (n=3), **NS**: Not significant, ^{a}: $P < 0.05$, ^{b}: $P < 0.01$, ^{c}: $P < 0.001$ compared to the standard Clamoxyl.

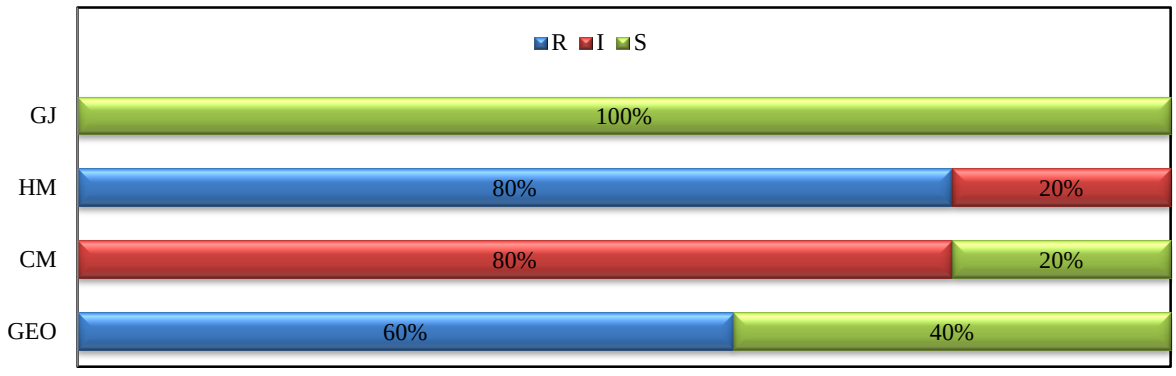

Figure 1. Percentage of resistant strains to garlic extracts. **R**: Resistant, **I**: Intermediate, **S**: Susceptible, **GEO**: Garlic essential Oil, **CM**: Cold macerate, **HM**: Hot macerate, **GJ**: Garlic juice.

MIC and MBC of garlic extracts

MIC, defined as the lowest concentration was able to prevent visible bacterial growth, and varied between 250 µl/ml and 300 µl/ml for garlic juice, and from 300 µl/ml to 500 µl/ml for the cold macerate (**Table 2**). Likewise, the MBC, which is the lowest concentration required to eradicate bacteria completely, ranged from 250 µl/ml to 300

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µl/ml for garlic juice, while it was between 250 µl/ml and 500 µl/ml for the cold macerate. Importantly, both garlic juice and cold macerate exhibited bactericidal effects on Escherichia coli at a concentration of 250 µl/ml, demonstrating their potential as effective antibacterial agents. MBC/MIC for all examined bacterial strains ranged from 1.0 to 1.66. According to the established interpretive criteria, this indicates that garlic juice has bactericidal properties against all the bacterial strains that were studied. In contrast, the cold macerate extract demonstrated bactericidal activity solely against *S. typhimurium* and *P. mirabilis*, while mostly showing bacteriostatic effects on the other strains.

Table 2. The values for the MICs and MBCs of garlic juice and cold macerate.

Strains	***E. coli***		***S. typhimurum***		***P. mirabilis***		***M. luteus***		***B.cereus***	
Extracts (µl/ml)	GJ	CM	GJ	CM	GJ	CM	GJ	CM	GJ	CM
MICs	250	500	300	300	500	300	300	300	300	0
MBCs	250	-	300	300	500	500	300	-	300	0
MBCs/ MICs	1	-	1	1	1	1,66	1	-	1	0
Antimicrobial effect	BC	-	BC	BC	BC	BC	BC	-	BC	-

GJ: Garlic juice, **CM**: Could macerate**, MIC**: Minimum inhibitory concentration, **MBC**: Minimum bactericide concentration, **BC**: Bactericid.

Microbiological analysis of poultry meat

Regarding thermo_tolerant coliforms and total coliforms, they were below the fixed standards; their average concentrations were 2.30 $\log_{10}$ and 3.54 $\log_{10}$ CFU/g, respectively. All the samples showed a value less than or equal to the norms fixed by Algerian standards.

Antimicrobial activity of garlic extracts incorporated in chicken meat

During the final period of storage (16 days), an increase in mesophilic aerobic microorganisms was noted. However, the garlic juice and cold macerate-supplemented treatments contained a statistically significantly low microbial load compared to the control, which registered 6.88 $\log_{10}$ CFU/g. A gradual decrease in the load is observed from the eighth to the sixteenth day of storage. Interestingly, garlic juice had a significant inhibitory effect on the growth of mesophilic aerobic microorganisms on day 4 of storage. Garlic juice and cold macerate were shown to reduce total coliform numbers compared to the control. This was especially evident with garlic juice, which had a total coliform load of 0 $\log_{10}$ CFU/g during storage (**Figure 2**). On the other hand, the cold macerate indicated a reduced inhibitory effect, with a microbial load of 1.95 $\log_{10}$ CFU/g in the storage period. Microbial load showed a very high decline on day 12 in the treatment. In addition, there was a notable variation in the mean counts of thermo-tolerant coliforms between the various treatments, determining the antimicrobial activity of garlic extracts during storage.

The percentages of bacterial groups' growth inhibition for each tested treatment are shown in **Figure 3**. As can be seen, the garlic juice presented an inhibitory effect on the mesophilic aerobic microorganism count and on both coliform groups with a percentage equal to 20% and 100%, respectively, in 16 days. The cold macerate demonstrated an inhibitory effect of 51.64%. A statistically significant difference in the average fecal coliforms count was observed among the treatments. The use of garlic extracts was more effective in controlling these microorganisms; the values were less than the criterion fixed by Algerian standards.

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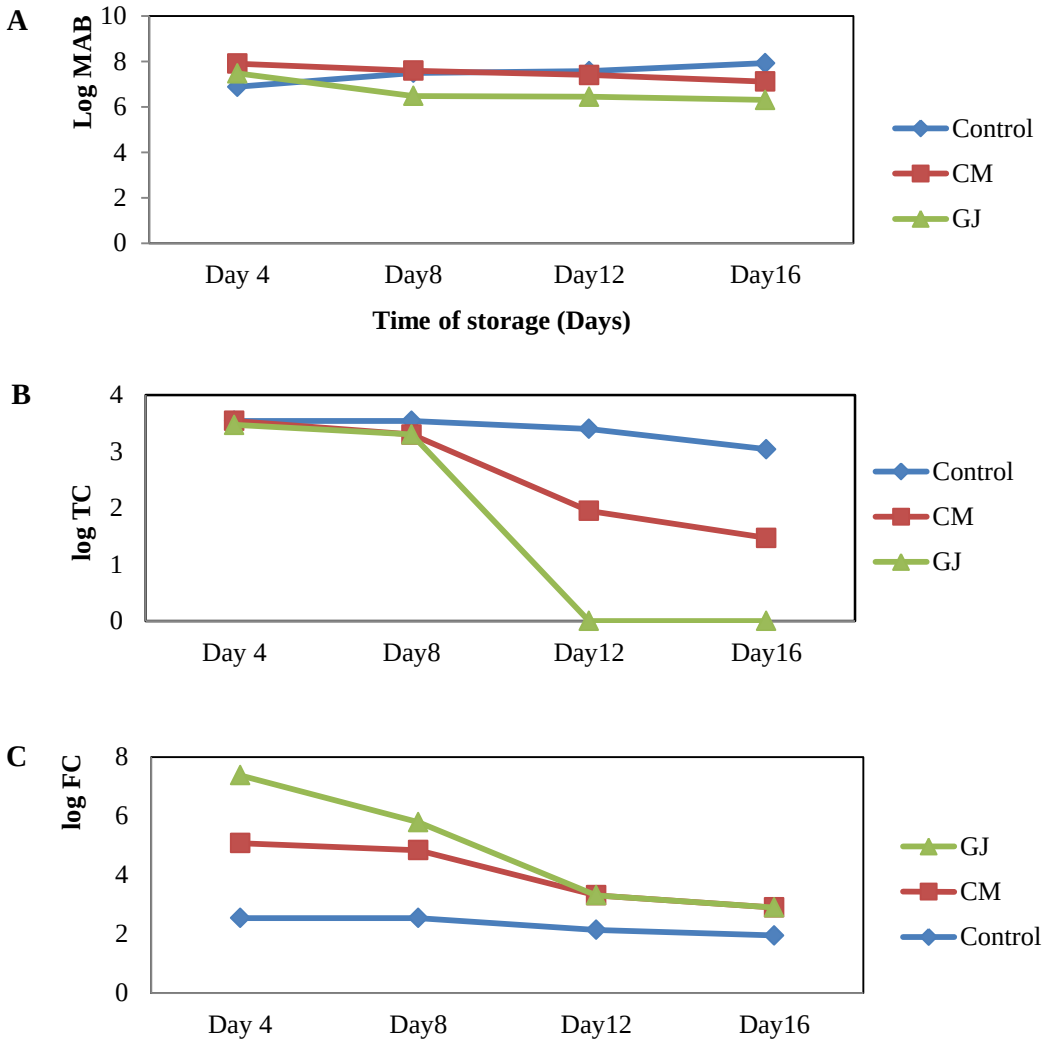

Figure 2. Effect of garlic juice and cold macerate on growth microbes, A) mesophilic aerobic bacteria (MAB), B) total coliform (TF), C) fecal coliform (FC), GJ: garlic juice, CM: cold macerate.

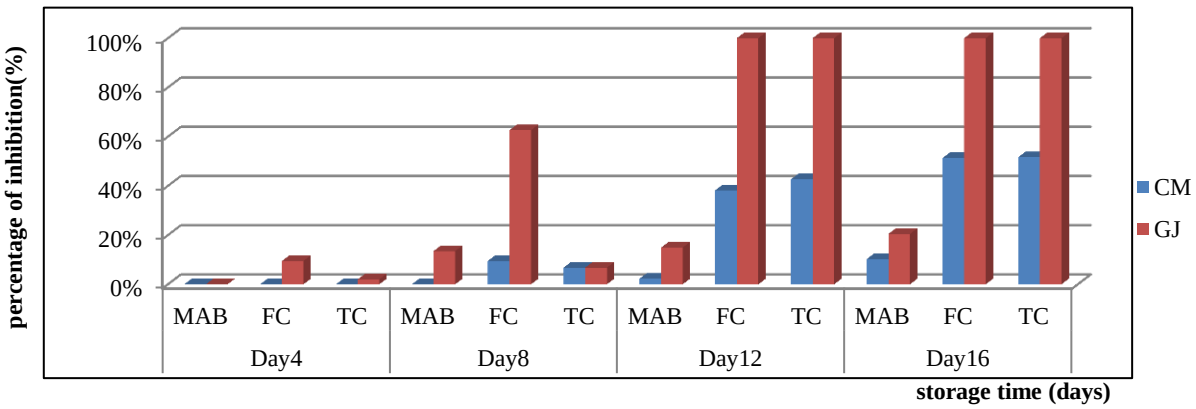

Figure 3. Percentage of bacterial growth inhibition in chicken meat after storage period. **MAB**: Mesophilic aerobic microorganism, **FC**: Fecal coliform, **TC**: Total coliform, **CM**: Cold macerate, **GJ**: Garlic juice.

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Discussion

The results of antibacterial activity revealed that *GJ* had a higher inhibitory effect on *Micrococcus luteus* (23.33±1.11 mm) and a lower inhibitory effect on *E. coli* (15.33±0.44 mm). The same result was obtained by Benmeddour (Benmeddour *et al.*, 2015), where *E. coli* appeared the most resistant to GJ with an inhibition zone between 8 and 15 mm. The result of this study revealed that HM of *A. sativum* had no antimicrobial activity against all the test organisms. Adetunde *et al*. studied the antimicrobial activity of heated extracts (ethanolic and aqueous) of garlic (*Allium sativum*) and ginger (*Zingiber officinale*) on selected pathogens using the agar-well diffusion assay method (Adetunde *et al.*, 2014). The results showed that neither the heated ethanolic extracts nor the heated aqueous extracts of *A. sativum* had antimicrobial activity against any of the test bacteria. On the other hand, the garlic essential oil had no activity against the test bacteria except for which exhibited an activity against *M. luteus* and *B. cereus* with inhibition equal to 19.83±0.77 and 29±3.33 mm, respectively. In the work of Benmeddour *et al.* (Benmeddour et al., 2015) , the antibacterial and antifungal effects in vitro of three species of the genus *Allium* were evaluated from fresh juices and essential oils of *A. cepa, fistulosum,* and *sativum*. The lowest antibacterial activity is obtained from oils.

Various garlic preparations have been shown to exhibit a wide spectrum of antibacterial activity against gram-negative and gram-positive bacteria, including species of *Escherichia, Salmonella, Staphylococcus, Streptococcus, Klebsiella, Proteus, Bacillus*, and *Clostridium*. Even acid-fast bacteria such as *Mycobacterium tuberculosis* are sensitive to garlic (Ankri and Mirelman, 1999, Mukhtar and Ghori, 2012, Ross *et al.*, 2001, Sivam *et al.*, 1997, Uchida *et al.*, 1975, Yusha'u *et al.*, 2008). have also confirmed that they are not only effective against bacteria but also possess antiviral and antifungal activity. Bajac *et al*. found that the antimicrobial activity of aqueous garlic extracts against Escherichia coli, expressed as the MIC, was estimated at 56.82 to 227.27 µl/ml, while the MBC ranged from 227 to 454.54 µl/ml depending on the garlic variety (Bajac *et al.*, 2018).

The results show that the means of the total aerobic mesophilic counts are 7.07 $\log_{10}$ CFU/g. The mean total viable counts are comparable to the values 6.64-6.91 $\log_{10}$ CFU/g, found by Amara *et al.* (Amara *et al.*, 1994), and higher than 4.52 $\log_{10}$ CFU/g, found by Selvan *et al*. (Selvan *et al.*, 2007). Higher total aerobic mesophilic counts in chicken meat were obtained by Nossair (Nossair *et al.*, 2015), who found that the mean value of APC was 4x 10^7±5 x 10^6 CFU in the examined 50 chicken breast meats. Regarding thermotolerant coliforms and total coliforms lower than the fixed standards, their average concentrations were 2.30 and 3.544 $\log_{10}$ CFU/g, respectively. According to Adu-Gyamfi (Adu-Gyamfi *et al.*, 2012), the mean total coliform counts for the supermarkets, local markets and farms were 3.80, 3.46, and 3.14 $\log_{10}$ CFU/g, respectively. Nearly similar results were reported by Mohammed, who mentioned that the mean value of total coliform count in chicken meat was 1.7 x 10 CFU/g (Mohammed *et al.*, 2019).

The use of garlic extracts was more effective in controlling these microorganisms; the values were less than the criterion fixed by Algerian standards. Hanane reported that the addition of 0.12 g/g of fresh garlic to Merguez sausages reduced the bacterial loads below the required norms (Hanane *et al.*, 2022). These results were in agreement with those previously reported (El-Khateib and Abd El-Rahman, 1987, Kalkan *et al.*, 2017, Mahros *et al.*, 2021). Sallam *et al*. reported that adding FG (30 g/kg) or garlic powder (9 g/kg) to chicken sausages significantly reduced their CPAs and, subsequently, extended their shelf life to 21 days (Sallam *et al.*, 2004). In ground beef with added garlic, the reduction in APCs, compared to control samples, can be attributed to organosulfur compounds and allicin from their precursor in garlic (Kyung, 2012). The main limitations of this

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study were the absence of chemical composition analysis of garlic juice and the verification of consumer approval regarding the concentration and flavor.

Conclusion

This study concluded that the addition of garlic extracts to poultry meat reduced their aerobic bacteria and coliform groups and increased their shelf life in refrigerated storage (at 4 °C for 16 days). Additionally, garlic juice had the best effect in comparison with other extracts, and 300 µl/ml/1 g showed the most potent effect. Therefore, garlic may be supplemented with meat products as a natural herb to lower their bacteria and extend the shelf life instead of some chemical preservatives.

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Authors' contributions

All the authors contributed to all parts of this work.

Conflict of interest

The authors declared no conflict of interests.

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