



OPEN Utilization of clove and cinnamon essential oils as an alternative to inhibit MDR and biofilm producing *E. coli* from raw chicken meat

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The microbial contamination and spoilage found in chicken meat is responsible for food-borne illnesses and outbreaks leading to hospitalizations. *E. coli* is the most commonly reported microorganism. The dissemination of bacterial strain with biofilm-formation ability and resistance to antimicrobials at the end of the food chain is a global concern; as well. Eco-friendly and novel means like essential oils are required to break these vicious patterns and ensure the longevity of quality food products. The study aimed to probe the prevalence, pattern of antimicrobial resistance and the biofilm formation ability in *E. coli* isolated from chicken meat samples. It also explored the antimicrobial and anti-biofilm formation ability of clove and cinnamon essential oils. 150 chicken meat samples from different localities of Karachi, Pakistan were isolated and identified by selective culturing and conventional microbiological techniques. Following antibiogram analysis, antibacterial activity of clove and cinnamon essential oils was evaluated. Putative biofilm production ability was also explored using the test tube, microplate reader, and scanning electron microscopy. Finally, the molecular characterization of potentially strong biofilm producers was done along with exploration of the pathogenic gene (*PapC*). 49 chicken meat samples out of 150 were contaminated with *E. coli*. 90% (44 isolates) of *E. coli* were multidrug resistant. 59.2% (29 isolates) were biofilm producers (BPs). Out of 29 BPs, nine (31%) were strong biofilm producers (SBPs). No significant correlations were observed between antimicrobial resistance and biofilm producing ability of *E. coli* isolates (p value ≥ 0.05). 40% of SBPs were inhibited when subjected to both clove (MIC: 250 to 500 $\mu\text{L/mL}$) and cinnamon (MIC: 62.5 $\mu\text{L/mL}$) EOs. Activity of both neat CO and CinO had no significant difference (p value ≥ 0.05). The identity of 3 SBPs (Strains: AR11E, AR12E and AR22E) were further confirmed by molecular identification (16SrRNA) and SEM revealed potential degradation of the bacterial cells with a reduction in count when treated with CinO and CO. Only one strain (AR22E) was positive for the *papC* gene. The prevalence of *E. coli* and strong-biofilm producers in retail chicken meat was not very high; however, the majority of the isolates were multi-drug resistant. Therefore, it is important to keep a tab on the prevalence of these commensal and pathogenic microorganisms in retail chicken meat since they are an exposure site close to the consumer. The use of alternative means like essential oils in poultry, meat and meat-products is a good strategy since they have proven efficacy against pathogenic *E. coli*.

Keywords *E. coli*; essential oil; poultry meat, Antimicrobial resistance, Biofilm, Hemolysin, 16SrRNA, SEM, *Pap C*

According to the World Bank and World Economic Outlook' 2023 both the global growth in population and improvement in per capita GDP in lower- and middle-income countries (LMICs) have favorably led to increased consumption of animal protein by consumers^{1,2}. Among the animal proteins poultry meat is the largest growing sector; its consumption has doubled from 67 million tons to approximately 131 million tons in a span of twenty years (2000 to 2020) which parallels its overall production of 121.6 million tons in 2021³. The major drivers of its consumption are its low production cost, affordability, lack of cultural or religious restriction and low-fat content as well as nutritional benefits^{4,5}.

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In natura chicken (*Gallus gallus domesticus*) meat is ideal for microbial contamination and spoilage which makes it a perishable food item with a short shelf-life. Not only high protein, high pH and water is conducive for the growth of the microorganisms but lipid content upon oxidation when exposed to environmental oxygen adversely affects the meat quality; as well⁶.

Biological hazards like viruses, bacteria and fungus are responsible for both extrinsic and intrinsic microbial contamination of chicken meat. Intrinsically, the meat (skeletal muscle) is mostly sterile with the exception of lymph nodes and is not the frequent cause of contamination⁷. Animal feathers, water, animal feed, human touch, factors associated with slaughterhouse facility like processing equipment, tools, surfaces etc., transportation and/or slaughtering of animals in wet markets at the butcher shop are the extrinsic causes of surface contamination of chicken meat. These contaminations are occurring across the production chain i.e. from farm to fork^{8–10}.

Food-borne illnesses and outbreak leads to hospitalization of about 128, 000 people out of which approximately 3000 succumb to death and about 48 million are reported sick in USA (CDC, 2018)¹¹. Similarly, out of the 43,400 reported cases of food-and water-borne outbreaks by European Food Safety Authority and the European Centre for Disease Prevention & Control (EFSA-ECDC, 2018) 385 of them were due to microbial contamination of meat and meat products¹². 25% of the 15.9 million reported gastroenteritis in Australia is because of contaminated food¹³. Most commonly reported species in the above-mentioned food-borne illnesses are *E. coli*, *Salmonella* and *Campylobacter spp*^{14,15}. The situation is grave in our part of the world with prevailing unhygienic practices observed in the food industry.

The quality of chicken meat sold in developing countries like Pakistan is of sub-standard quality due to the poor sanitation/hygiene conditions along with conventional ways of processing, handling and selling. These factors are mostly attributed to the substantial external surface meat contamination which not only decreases the shelf-life because of spoilage rather, also increases the risk of food-borne infections and diseases. Besides food spoilage and short shelf-life, the spread of disease-causing pathogens and dissemination of bacterial strain resistant to number of antimicrobials at the end of the food chain is of global concern. Antimicrobial resistance (AMR), according to WHO, is one of the top 10 global threats faced by humanity which is responsible for the growing food insecurities, economic losses and human morbidity.

Extrinsic factors enumerated in the aforementioned paragraphs serve as the nidus for growth of a vast array of micro-organisms on the surface of chicken meat. Among these micro-organisms *Campylobacter*, *Bacillus cereus*, *Listeria monocytogens*, *E. coli*, *S. aureus*, *Salmonella spp*, *Clostridium spp*, *Pseudomonas* and *Aeromonas hydrophila* are pathogenic^{16–18}. In 2010 Ali et al. demonstrated that 84% of 250 raw meat samples were contaminated with the following bacterial species: *S. aureus*, *Bacillus subtilis*, *Enterobacter*, *E. coli*, *Listeria*, *Brucella*, *Salmonella*, *Shigella* and *Klebsiella*. 66% of the aforementioned isolates were not only potentially pathogenic rather, showed resistance to cefaclor, amoxicillin, novobiocin and ampicillin. Furthermore, the isolated species were found to be biofilm producers; as well¹⁹.

In the face of rising antibiotic resistance (ABR) and side effects of chemical preservatives use of either of these is not a wise choice for the safe and quality supply of food commodities^{20,21}. However, the use of natural compounds extracted from the plants hold a promising potential to combat microbial contamination and spoilage thus ensuring both the safety and quality in food industry^{22–24}. The plants during their metabolic cycle synthesize a plethora of secondary metabolites which help them to withstand harsher environmental conditions. The mixture of these volatile secondary metabolites is termed as essential oil (EO)^{22,25–27}.

Innately EOs have antibacterial, antioxidant, antifungal and antiviral activities. The antimicrobial activities have been attributed to their ability to disrupt the DNA replication, rupture either or both the cell wall and membrane, induce oxidative stress-mediated apoptosis and inhibit biofilm formation^{25,28–31}. Moreover, their low or non-toxicity against vertebrates and biodegradability makes them both environmental friendly and safe for human use. Therefore, the Food and Drug Administration (FDA) of United States recognizes them as safe substances^{32–34}. It has been cited in the literature that EO extracted from several herbs like cumin, rosemary, basil and thyme etc. have strong antibacterial activity against food-borne pathogens which has a promising effect on the shelf-life of chicken meat by decreasing the spoilage and thereby are good alternatives as compared to the synthetic antibacterial agents. Furthermore, they cannot only be used as novel-agents against drug resistant bacteria rather, as an efficient source to combat the biofilm formation; as well^{35–38}.

Therefore, in the face of growing consumer concern regarding the increasing number of food-borne illnesses and the un-safe use of synthetic antimicrobials which are also precipitating AMR we conducted this study to (1) evaluate the prevalence of MDR and biofilm forming *E. coli* from chicken meat and (2) assess the antimicrobial and anti-biofilm potential of clove and cinnamon EOs. This was done with the objective of developing an insight into incorporating antimicrobial compounds of essential oils in place of chemical preservatives in food packaging to improve the shelf-life and quality of chicken meat. This study is a multifaceted investigation regarding the prevalence and characterization of *E. coli* in raw chicken meat, which is further augmented by AST, natural antimicrobials assessment, morphological ultrastructure analysis of biofilm and molecular profiling. Secondly, unlike previous studies, this study used oil concentrations range from 62.5 to 1000 µg/mL providing evidence into the promising activity of cinnamon and clove essential oils against *E. coli spp*. isolated from the raw chicken meat.

Methodology

Materials/Reagents

Gram staining reagents were purchased from Merck KGaA Germany. Essential oils i.e. clove oil.

(CO) and cinnamon oil (CinO) were acquired from Haque planters (Karachi, Pakistan). All the other reagents, media and antibiotic discs used in the study were the products of Oxoid Ltd Basingstoke, UK. American Type and Culture Collection (ATCC culture 25922) was obtained from Musaji Adam & Sons (Karachi, Pakistan) and DNA purification kit was from GJC[®] Bacterial DNA Purification Kit.

Sample collection and transportation

A total of 150 fresh chicken breast samples were purchased from the various wet markets of urban localities of Karachi, Pakistan. The collection was done over a period of 9 months i.e. from March to November 2021. The purchased samples were collected in pre-autoclaved conical flask (250 mL) and transported immediately in the icebox (0 to 4 °C) to avoid spoilage. The collected samples were processed freshly and further analyzed at microbiology laboratory, University of Karachi.

Isolation and identification of *Escherichia coli*

Isolation and identification

For the isolation of *Escherichia coli*, 25 g of chicken breast meat from each sample were cut and minced. The minced meat was then homogenized in 225 mL of pre-autoclaved peptone water (Oxoid Ltd Basingstoke, UK). The homogenate was 10-fold serially diluted in 0.9% saline solution. 0.1 mL (100 µL) of 10^{-2} dilution was spread on to the selective agar i.e. eosin methylene blue (EMB) agar (Oxoid Ltd Basingstoke, UK) plates and incubated at 37 °C overnight³⁹. A positive control of *Escherichia coli* (ATCC 25922) was also run in parallel. Next day, plates were observed for the isolates producing green metallic sheen on EMB (Oxoid Ltd Basingstoke, UK) and were further confirmed by biochemical tests and microscopy.

Biochemical and microscopy

The gram staining (Merck KGaA Germany) and examination via the oil immersion lens (100X) of compound light microscope was used to delineate the microscopic characteristic of the isolates of *E. coli*. The identification of *E. coli* was further elaborated by the following battery of biochemical tests: Indole, Methyl Red, Voges-Proskauer, and Citrate (IMViC), TSI (Triple Sugar Iron) and SIM (Sulphur Indole Motility) (Oxoid Ltd Basingstoke, UK). The isolates confirmed by both microscopy and biochemical tests were sub-cultured in heart infusion (HI) (Oxoid Ltd Basingstoke, UK) agar slants for subsequent analysis⁴⁰.

In vitro pathogenicity of *E. coli*

The pathogenicity of 49 isolates of *E. coli* were evaluated using the following methods.

Hemolysin activity

The hemolytic ability of the isolates was assessed using nutrient agar (Oxoid Ltd Basingstoke, UK) supplemented with sheep blood (5%). The said plates were streaked with *E. coli* isolates and incubated overnight at 37 °C. After 24 h, the plates were observed and stratified as β -hemolytic (complete lysis of RBCs), α -hemolytic (partial) and γ -hemolytic organisms (no lysis)⁴¹.

Biofilm formation

Following the assessment of hemolysin activity, the pathogenicity of *E. coli* isolates was further evaluated for biofilm formation using test tube and microplate method.

Test tube method The reference strain: ATCC *E. coli* 25,922 along with all the identified isolates were screened for biofilm formation. The culture of each isolate was inoculated in labeled test tubes containing 10 mL HI (Oxoid Ltd Basingstoke, UK) and incubated for 24 h at 37 °C. Blank HI (Oxoid Ltd Basingstoke, UK) tube was run as a negative control. Next day, following aspiration of content of the tubes they were washed thrice with phosphate buffer saline (PBS). Aspirated and washed tube were stained with 0.1% crystal violet (CV) solution for 15 min. Following staining, CV was decanted and the tubes were left to air dry in an upright position. The visibility of the ring along the circumference of the tube at solid-liquid interface were considered as biofilm producers. (BPs)^{42,43}.

Microplate reader All the 49 isolates of *E. coli* were allowed to form a biofilm on pre-sterilized 96 well flat bottom polystyrene microtiter plates. The bacterial colony of each isolate was inoculated in 10 mL of HI (Oxoid Ltd Basingstoke, UK) and incubated at 37 °C for 24 h. The following day, using the sterile saline the turbidity was re-adjusted to match the 0.5 McFarland standards (1×10^8), and HI (Oxoid Ltd Basingstoke, UK) was supplemented with 1% glucose. The bacterial suspensions were 200-fold diluted to obtain 5×10^5 CFU/mL.

Briefly, 190 µL of fresh HI (Oxoid Ltd Basingstoke, UK) was dispensed in the labeled wells of 96 well plates. 10 µL of each test isolate was added in the correspondingly labeled wells along with the one positive control (biofilm producer; *E. coli* ATCC 25922). A negative control with no bacterial suspension was also ran in parallel. In order to avoid evaporation, the plates were covered with lid and allowed to incubate in a static position. After 48 h of incubation, the suspension was carefully decanted to avoid disruption of the biofilm. The wells were washed twice with phosphate buffer saline (PBS) and air-dried. Following drying, the plates were stained with 0.1% CV for 15–20 min. The excess stain was removed by washing with distilled water. The bound CV to adherent cells were eluted using 96% ethanol and placed in a Microplate reader (Thermo Scientific, Multi-Scan FC) to measure the optical density (OD) at 570 nm^{44,45}. Each isolate (i.e. 49) was run in triplicates ($n = 3$) and their average OD values \pm standard deviation (SD) was calculated. To interpret the production of biofilm, cutoff value of optical density (ODc) was calculated using the following formula.

$$\text{ODc} = \text{average OD of negative control} + (3 \times \text{SD of negative control}).$$

The classification of *E. coli* isolates as non- biofilm producers (NBPs), weak-BPs (WBPs), moderate-BPs (MBPs), and strong-BPs (SBPs) was based on ODc value. For example, $\text{OD} \leq \text{ODc}$, $\text{ODc} < \text{OD} \leq 2 \times \text{ODc}$, $2 \times \text{ODc} < \text{OD} \leq 4 \times \text{ODc}$, $4 \times \text{ODc} < \text{OD}$ was categorized as NBPs, WBPs, MBPs, and SBPs respectively.

Antimicrobial susceptibility testing (AST)

The antibiotics susceptibility pattern of the confirmed isolates was determined by employing the Kirby-Bauer disk diffusion method following a standard protocol. For the AST, fresh cultures of *E. coli* isolates matched with 0.5 McFarland standards were inoculated on Mueller Hinton Agar (MHA) (Oxoid Ltd Basingstoke, UK) plates along with the commercially available antibiotic discs (Oxoid Ltd Basingstoke, UK): chloramphenicol 30 µg, tetracycline 30 µg, gentamicin 10 µg, ciprofloxacin 5 µg, amoxycillin 30 µg, cefoxitin 30 µg, streptomycin 10 µg and erythromycin 15 µg. Following inoculation, they were incubated at 37 °C for 24 h. After incubation, the zone of inhibition (ZOI) around the disc was measured in millimeters (mm). According to the CLSI guidelines, susceptibility patterns were considered as sensitive, resistant, and intermediate based on the size (mm) of ZOI^{46,47}.

In-Vitro antimicrobial evaluation of clove and cinnamon essential oil

The following nine SBPs (4 × OD < OD): A12E, A14E, A18E, A26E, A34E, AR11E, AR12E, AR18E and AR22E were selected to evaluate the antimicrobial activity of clove and cinnamon essential oil using the agar-well diffusion method as well as to determine their minimum inhibitory concentrations (MICs) through the broth dilution method.

Minimum inhibitory concentration

MIC was determined to evaluate the effectiveness of EOs against 9 SBPs by the method previously described⁴⁸. The cultures of SBPs were revived by inoculating them in HI (Oxoid Ltd Basingstoke, UK) agar plates under aseptic conditions. The broth dilution method was performed in order to do serial dilution of EOs using 40% DMSO and the two-fold decreasing concentration of EOs were used. Five concentrations were prepared within the range of 62.5 µL/mL to 1000 µL/mL. Autoclaved and labeled tubes were filled with 1000 µL of MHB followed by the addition of EO to achieve the following final concentrations: 1000 µL/mL (Tube 1), 500 µL/mL (Tube 2), 250 µL/mL (Tube 3), 125 µL/mL (Tube 4), and 62.5 µL/mL (Tube 5). This was followed by the addition of 10 µL of freshly grown culture (matched with 0.5 McFarland concentration) along with the one positive control. The MIC of EOs required to inhibit the respective isolate were compared by observing the decrease in turbidity following the overnight incubation⁴⁹.

Agar well diffusion method

The standardized bacterial suspensions were spread uniformly on MHA (Oxoid Ltd Basingstoke, UK) plates following this equally spaced wells were made with a sterile borer (6 mm diameter)⁴⁹. The wells were labeled with codes assigned to 9 SBPs and filled with 20 µL of essential oil. 20 µL of 40% dimethyl sulfoxide (DMSO) was used as a negative control. For the sake of comparison, a positive biofilm producer; ATCC 25922 strain was also run simultaneously. Next day, the zone of inhibitions was calculated around each well, and their statistical differences were evaluated. All the experiments were performed in triplicates ($n = 3$) for each of the 49 isolates and one positive control. The data were expressed as the mean ± SD.

Scanning electron microscopy (SEM)

The morphological features of the three strong biofilm producers (AR11E, AR12E and AR22E) were obtained using scanning electron microscope (Thermo Fisher Scientific, Model Apreo 2 LoVac, USA) at International Center for Chemical and Biological Sciences (ICCBS), Karachi. The sub-culturing and enrichment were performed using the methods mentioned previously in the screening of biofilm producers. 0.1 mL (100 µL) of the culture was added in a sterile petri plate containing 20 mL of HI broth along with sub- and minimum-inhibitory concentration of EOs (CO and CiO) to delineate the architectural differences after treatment. The sterile glass coverslips (20 × 20 mm) were gently dipped in each labeled (AR11E, AR12E and AR22E) petri plate. For the sake of visualization of biofilms, control without any antibacterial agent was also run to observe the extracellular polymeric substance (EPS) formation by the isolates. Finally, the plates were incubated for 48 h at 37 °C. After the specified time of incubation the coverslips were carefully removed from the prepared suspension with the help of sterile forceps. They were placed on another empty sterile plate and rinsed with PBS to eliminate the non-adherent cells. Subsequently for the fixation of biofilm Methacarn (60% methanol, 30% chloroform, and 10% glacial acetic acid) solution was used. After fixation the coverslips were serially dehydrated in ethanol solution of decreasing strength (i.e. 70%, 80%, and 90%) for 15 min each. Coverslips were then dried in a hot air oven to prevent any distortion of biofilm. The dried coverslips were coated up to 153 Å with gold/palladium using a sputter coater JEOL (SC7620-Quorum Technologies) equipped with an ion sputtering device (model EX-54175JMU; JEOL, Tokyo, Japan)^{50,51}. Following aforementioned preparation of the isolates, the morphological alterations in the bacterial cell before and after treatment were observed under SEM. Final images were taken at 5000, 10,000 and 20000X.

Molecular identification of *E. coli*: PCR

16SrRNA

For the rapid and accurate identification of *E. coli*, three SBPs (i.e. AR12E, AR22E and AR11E) with the highest OD values at 570 nm were selected. The extracted DNA was amplified for 16SrRNA through conventional polymerase chain reaction (PCR) and DNA sequencing was done through Sanger method. Genomic DNA of selected isolates were extracted using GJC[®] Bacterial DNA Purification Kit followed by PCR amplification targeting the 16SrRNA gene using universal primers: Forward F27 5'-AGAGTTTGATCATGGCTCAG-3'; Reverse R1488 5'-CGGTTACCTTGTTAGGACTTCACC-3'). The 20 µL of both the forward and reverse primers along with 7 µL sample DNA were added into the master mix. The final volume of 35 µL was achieved by adding appropriate volume of sterilized distilled water. The mixture was vortexed, and micro centrifuged for

few seconds and finally PCR mixture was run in Veriti 96 well thermal cycler (Applied biosystems-Singapore) under following conditions: initial denaturation (94 °C for 5 min), followed by 30 cycles of denaturation (94 °C for 1 min), annealing (63 °C for 1 min), and extension (72 °C for 1 min), followed by a final extension (72 °C for 10 min). The amplified genes were run on agarose gel and visualized through transilluminator and the obtained electropherogram was analyzed for the quality of amplified product⁵². The PCR products were sent to Macrogen (South Korea) for DNA sequencing. Finally, the sequences were subjected to Basic Logic Alignment Search Tool (BLAST) in order to find the homologous sequences within the database.

A cladogram depicting the relationships among the strains along with their respective reference strains was reconstructed by using the best evolutionary model; Jukes and Cantor model (JC+G). The neighbor Joining tree based on 16SrRNA sequences of isolated strain with 1000 bootstrap replicates was generated. The tree was rooted with 16SrRNA sequences of *E. coli* strains (AR11E, AR12E and AR22E) along with *E. faecalis* strains as the outgroups.

PapC gene

Three out of nine SBPs were recruited for detection of biofilm forming gene: *PapC*. The gene was identified by PCR amplification. DNA was extracted from three SBPs and PCR mixture was prepared. The final volume of 25 µL was made in PCR tubes by mixing 12 µL master mix, 1 µL each of forward (5'- GACGGCTGTACTG CAGGGTGTGGCG'3') and reverse (5'- TCCTTTCTGCAGGGATGCAATA-3'), 3 µL of DNA template and approximately 8 µL of DNase free water. The target gene of 328bps was amplified⁵². The PCR protocol for 30 cycles were followed as: initial denaturation at 94°C followed by denaturation at 96°C for 1 min, annealing, at 63°C for 30 s, extension at 72°C for 3 min and final extension at 72°C for 7 min.

Statistical analysis

Differences between sizes of zones of inhibition were represented as mean ± standard deviation. The Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparison among the different categorized biofilm producers was used. Biserical Correlation (Pearson) was used to determine the correlation between different antibiotics and biofilm formation, *r* value closer to ± 1 shows the stronger correlation. The analytical results were considered statistically significant with a *p* value of < 0.05. GraphPad Prism 9 (GraphPad Software Inc., La Jolly, CA, United States) was used for all the statistical analysis.

Results

Isolation and colonial characteristics of *E. coli*

Forty-nine (32.6%) chicken meat samples out of 150 showed green metallic sheen on EMB and pink color colonies on MacConkey's agar indicating the presence of *E. coli*. Gram-negative rods in scattered confirmation as expected from *Escherichia* isolates were also observed at oil immersion lens under the microscope. Concurrently, IMViC and catalase test further confirmed that isolates of *E. coli* were positive for catalase, indole, and MR while testing negative for the VP. Furthermore, a small number of isolates, i.e. 7, were observed to be positive for citrate whereas 48 isolates (97.95%) demonstrated motility (Supplementary Table.1 S). Similarly, in the triple sugar iron test, all isolates (100%) showed a yellow slant, yellow butt, and gas production.

In vitro pathogenicity of *E. coli*

Hemolysin assay

14.28%⁷ of the *E. coli* isolates were hemolysin producers. Among which 10.2%⁵ and 4.08%² exhibited beta and alpha hemolysis; respectively (Figure.1 A & B).

Screening of biofilm producers

In the screening of biofilm analysis, the number of producers can vary depending on the incubation time. Among the *E. coli* isolates, 9 (18.3%) of them exhibited visible biofilm formation at the solid-liquid interface within the test-tube after 24 h whereas 15 (30.61%) were observed after 48 h of incubation (Supplementary Table.2 S). The color intensity of crystal violet retained in the adherent layer was estimated as negative (-), weak (+) and strong (++) (Figure.1 C and 1D). The results of qualitative analysis of biofilm were in agreement with the quantitative analysis where increased mean OD values suggested a high amount of biofilm formation and vice versa (Figure.2 A). Additionally, in the microtiter plate out of 49 *E. coli* isolates 59.2% isolates (Fig. 1E) were observed as BPs whereas, remaining 20 (40.8%) were NBPs. Out of 29; 9 (18.3%), 6 (12.2%) and 14 (28.5%) were observed as SBPs, MBPs and WBPs; respectively (Figure. 2B). The result for ATCC positive control strain was also observed as strong BPs due to the thick adherent layer and increased OD value ($4 \times \text{ODc} < \text{OD}$ i.e. > 0.62). The mean ± SD values of the BPs are presented in Supplementary Table.2 S.

Antimicrobial susceptibility testing (AST)

A significant proportion, i.e. 67.35% of the *E. coli* isolates demonstrated sensitivity to gentamicin followed by ciprofloxacin (28.57%) and tetracycline (26.53%). Whereas lower sensitivity rates were observed against other antibiotics: streptomycin (22.40%), amoxicillin-clavulanate (12.24%), chloramphenicol (8.16%) and erythromycin (6%) (Table 1). Compared to these, cefoxitin and erythromycin were the least effective antibiotics exhibiting highest resistance among 97.96% and 85.71% of isolates; respectively. Moreover, a crucial proportion (81.63%) of isolates were found resistant to chloramphenicol and amoxicillin-clavulanate followed by ciprofloxacin, tetracycline, and streptomycin with 51.02% each. Briefly, it was observed that at least 2 isolates (4.08%) were resistant to one antibiotic while the majority of the isolates (14 i.e. 28.57%) showed resistance to six antibiotics (Figure.3). Supplementary Table.3 S presents the mean ± SD values of antibiotic tested against each of the 49 isolates.

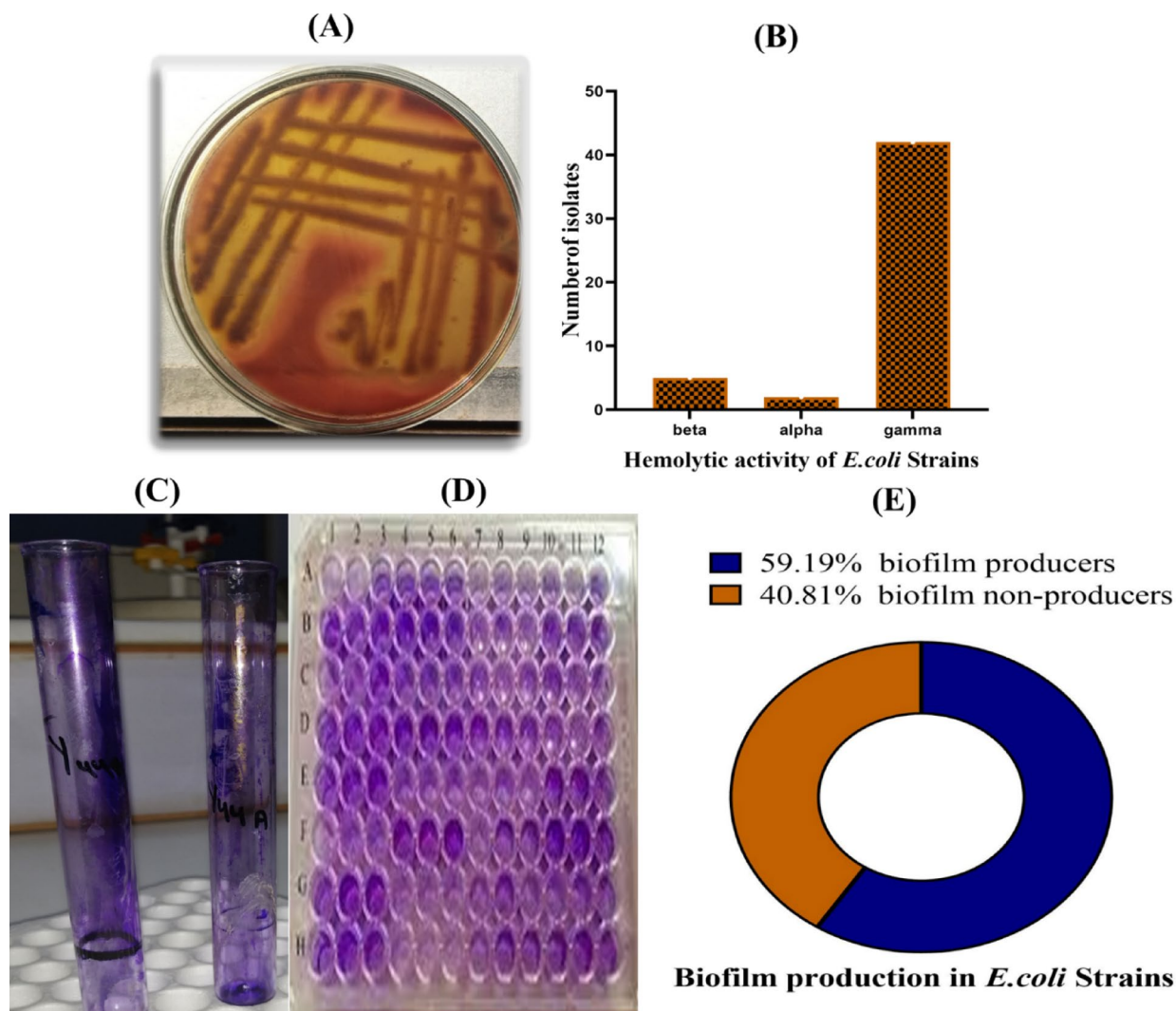


Fig. 1. Hemolytic activity and Biofilm Formation in *E. coli* Isolates. **(A)** *E. coli* isolates isolated from raw chicken meat were screened for hemolysis by inoculating on Blood agar plate. **(B)** The histogram shows the frequency of alpha, beta and gamma hemolytic isolates. The clear, partial and no lysis of RBCs represents β , α and γ hemolysis, respectively. **(C)** Biofilm production was screened by test tube and **(D)** microtiter plate method. **(E)** The donut graph shows the frequency of biofilm producers and non-producers.

Moreover, SBPs were observed to have an increased resistance to antibiotics with a mean resistance value of 73.61 ± 8.642 . No significant correlations were observed between antimicrobial resistance and biofilm producing ability of *E. coli* isolates (p value ≥ 0.05) (Table 2). However, the virulence trait of biofilm demonstrated moderate and positive correlations with one antibiotic i.e. erythromycin ($r = 0.5995$, p value ≥ 0.05).

In-Vitro antimicrobial evaluation of clove and cinnamon essential oil

Minimal inhibitory concentration (MIC)

MIC of the CO and CinO by broth dilution method revealed that both oils are effective against the tested nine isolates which were SBPs and MDR; as well. (Figure 4). The activity of CinO was promising against all the isolates, i.e. 40% of the isolates were inhibited at the lowest concentration of $62.5 \mu\text{L/mL}$. Compared to CinO, the inhibition pattern of CO was different as most of the isolates were inhibited either at 250 or $500 \mu\text{L/mL}$. Among all the tested isolates AR11E was the most resistant exhibiting growth inhibition at the higher concentration of both CinO ($500 \mu\text{L/mL}$) and CO ($1000 \mu\text{L/mL}$).

Essential oil activity by agar well diffusion method

When the *E. coli* isolates were tested with neat EOs by agar well diffusion method, results demonstrated the higher level of efficacy of both the CO and CinO with no significant statistical difference ($p > 0.05$) (Table 3).

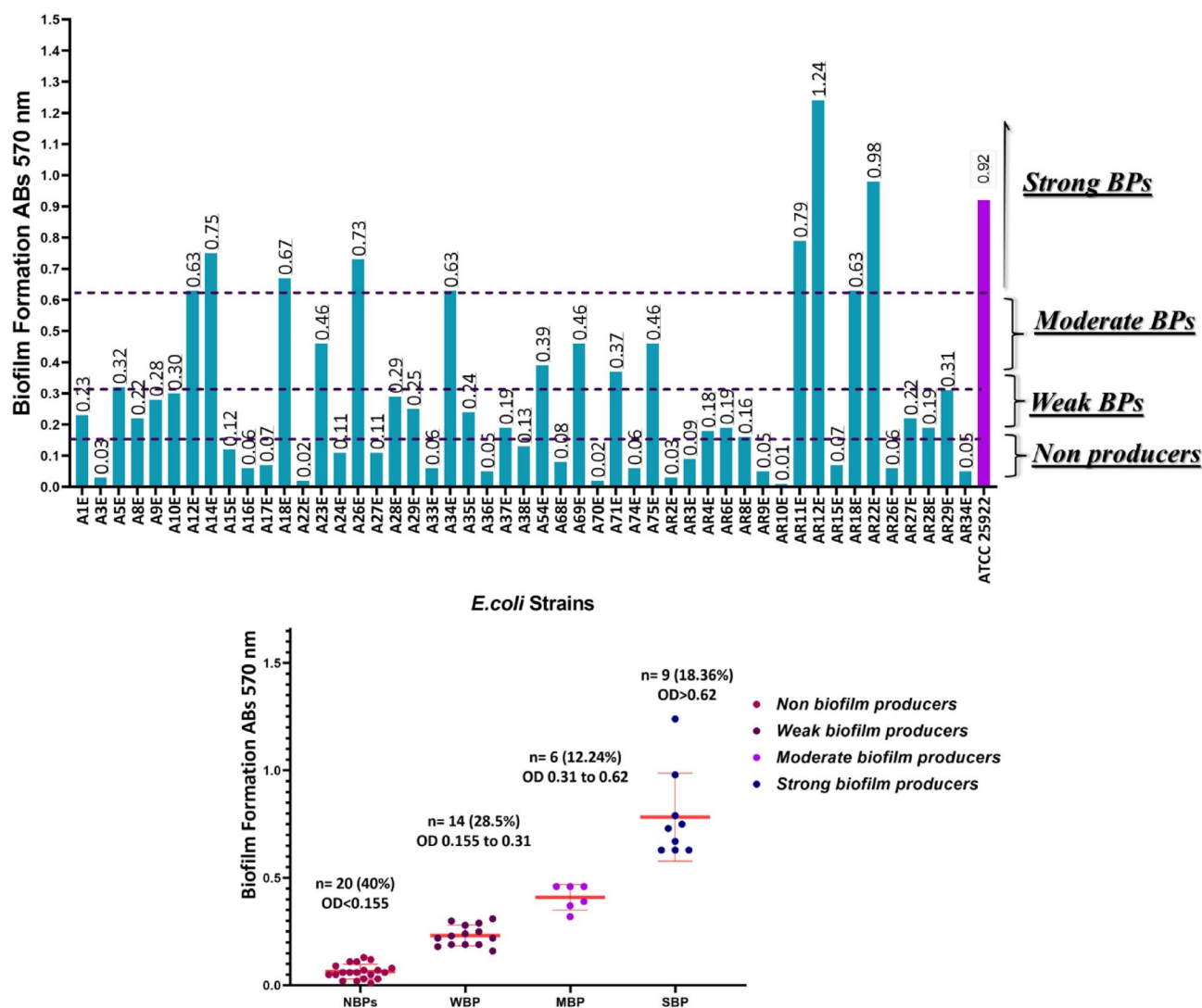


Fig. 2. Screening of Biofilm Formation by Microtiter Plate Method. **(A)** Absorbance values (ABs) of 49 strains of *E. coli* were quantitatively measured using 96 well plate on a microplate reader at a wavelength of 570 nm. Data were presented as mean \pm SD of three independent experiments performed in triplicate. OD values ≥ 0.62 , between 0.31 and 0.62, 0.155 and 0.31 and < 0.155 represent strong, moderate, weak and non-biofilm producers; respectively. **(B)** Graph showing frequency comparison of weak (W), moderate (M), and strong (S) biofilm producing *E. coli* isolates according to their OD values. The small circles with the distinguishing colors indicate the data points. The large and small horizontal lines in the graphs denote data's mean and standard deviation; respectively. p -value ≤ 0.05 was considered statistically significant.

<i>E. coli</i>	TE	CIP	CHL	FOX	E	STR	CN	AMC
Resistant	51.02%	51.02%	81.63%	97.96%	85.71%	51.02%	26.53%	81.63%
Sensitive	26.53%	28.57%	8.16%	0.00%	6%	22.40%	67.35%	12.24%
Intermediate	22%	20.41%	10.20%	2.04%	8.16%	26.53%	6.12%	6.12%

Table 1. Antimicrobial Susceptibility Pattern of 49 isolated strains of *E. coli* against the following eight antibiotics * TE: Tetracycline, CIP: Ciprofloxacin, CHL: Chloramphenicol, FOX: Cefoxitin, E: Erythromycin, STR: Streptomycin, CN: Gentamicin and AMC: Amoxicillin-clavulanate.

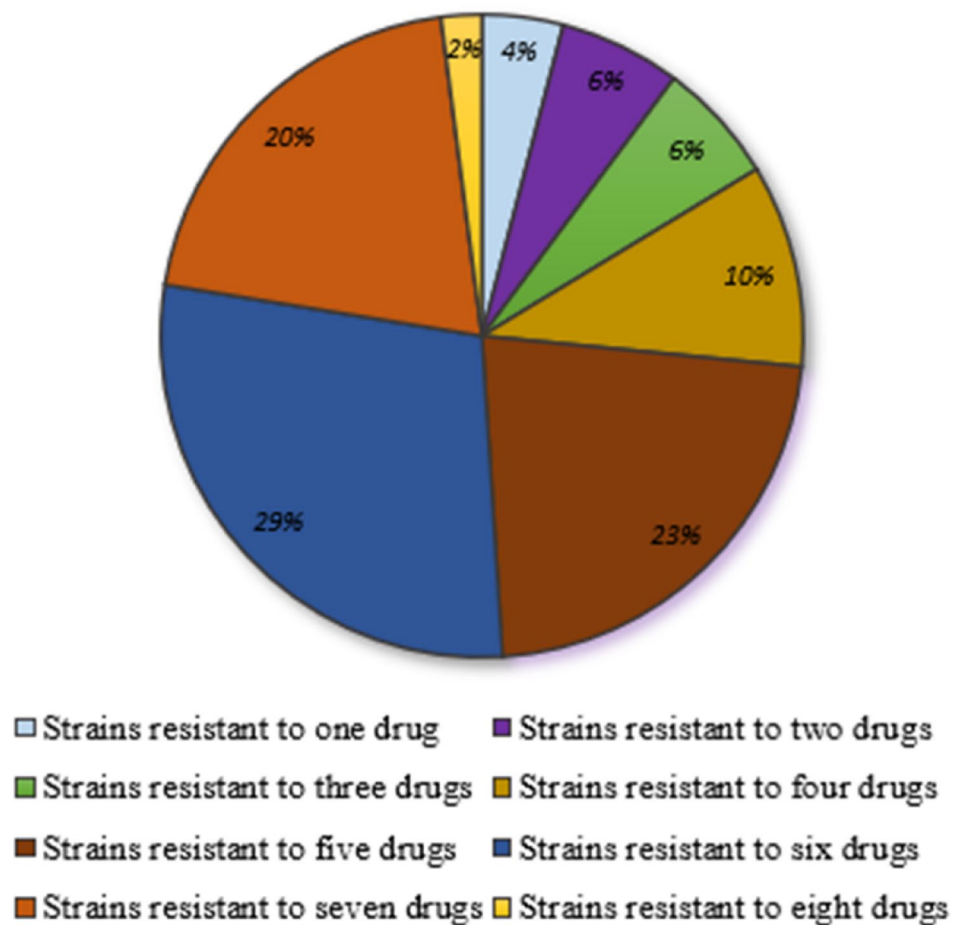


Fig. 3. Depicts the pattern of drug resistance against eight antibiotics in the *E. coli* isolates from raw chicken breast. 90% of the isolates were multidrug resistant out of which 29% were not susceptible to at least six antibiotics whereas; 2% showed resistance to all the antibiotics tested.

Antibiotics	Strong biofilm producers (9)				Correlation coefficient“r”	p- value
	S	%	R	%		
Chloramphenicol	1	11.11%	8	88.88%	-0.2313	0.5493
Tetracycline	4	44.44%	5	55.55%	-0.3009	0.4314
Gentamicin	6	66.66%	3	33.33%	-0.4203	0.2600
Amoxicillin-clavulanate	1	11.11%	8	88.88%	-0.2505	0.5156
Streptomycin	4	44.44%	5	55.55%	0.3978	0.2891
Erythromycin	0	0%	9	100%	0.5995	0.0879
Ciprofloxacin	3	33.33%	6	66.66%	0.3224	0.3974
Cefoxitin	0	0.00%	9	100%	0.0555	0.8871
Mean±SD		26.39±8.641		73.61±8.642		

Table 2. Correlation Between the Biofilm Production and Antibiotic Resistance in *E. coli* Strains. *% Percentage, S Sensitive, R Resistance.

Scanning electron microscopy (SEM)

The results of SEM were consistent with the findings observed through other methods of screening (i.e. tube and microplate). Formation of biofilm and its degradation following the treatment with CO and CinO at their MIC and sub-inhibitory MIC was seen in three selected SBPs: AR11E, AR12E and AR22E. The components of EOs damaged the cell due to which the intracellular constituent were released and observed in SEM images (Figure.5, 6 & 7). Consistent with the findings of agar well diffusion the inhibitory effect of cinnamon oil was more pronounced compared to clove oil as it almost eradicated the formed biofilm.

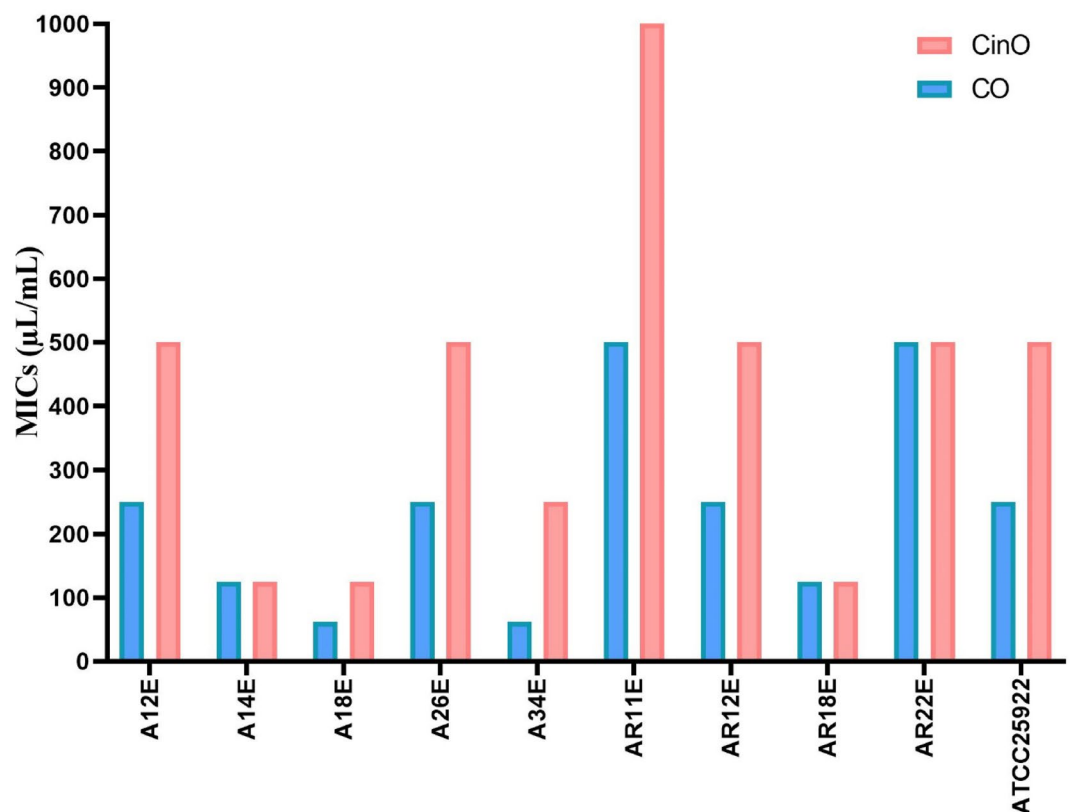


Fig. 4. Minimal Inhibitory Concentration of CO and CinO Against *E. coli* isolates. Results of the broth dilution assay performed on 9 Strong biofilm producers; A12E, A14E, A18E, A26E, A34E, AR11E, AR12E, AR18E and AR22E and 1 ATCC culture of *E. coli* are shown. The red and blue bars indicate the MIC values of CinO and CO respectively.

SBPs	OD value at 570 nm	Clove oil (20 μ L)	Cinnamon oil (20 μ L)	<i>p</i> value
A12E	0.65	27 \pm 0.029	31 \pm .081	0.1563
A14E	0.75	24 \pm 0.040	25 \pm 0.044	
A18E	0.67	22 \pm 0.041	30 \pm 0.009	
A26E	0.73	29 \pm 0.041	25 \pm 0.020	
A34E	0.63	28 \pm 0.020	28 \pm 0.047	
AR11E	0.79	31 \pm 0.141	27 \pm 0.020	
AR12E	1.24	23 \pm 0.014	30 \pm 0.062	
AR18E	0.63	21 \pm 0.047	24 \pm 0.047	
AR22E	0.98	24 \pm 0.0169	32 \pm 0.047	
ATCC 25922	0.92	23 \pm 0.040	26 \pm 0.009	

Table 3. Antimicrobial activity (ZOI) of pure 20 μ L EO (undiluted) against SBPs. The OD values of the 9 SBPs (refer to Figure.1; as well). The Wilcoxon signed rank test was used on ZOI values obtained from CO and CinO to assess the statistical difference between them.

Molecular identification and characterization of *E. coli*: polymerase chain reaction (PCR)

Molecular identification

Distinct and unambiguous bands of isolated *E. coli* strains were observed. The band was approximately 1.6 Kbp which indicates complete amplification of target gene i.e. 16SrRNA (Figure.8 A). The obtained electropherograms exhibited minimum noise and clear base calls. Blast results revealed the homology of sequenced 16SrRNA of three strains with *Escherichia coli*. Strong query coverage and query identity were observed (Table 4) supporting that isolated strains are *E. coli*. Furthermore, reconstructed cladograms showed strong agreement with the reference strain of respective species thus confirming the identification of *E. coli* strains (Figure.8B). The acquired nucleotide sequences of 16SrRNA (FASTA format) of AR11E, AR12E and AR22E strains were submitted to NCBI, and the following accessioned numbers were allocated: PP504317, PP504482 and PP504481; respectively.

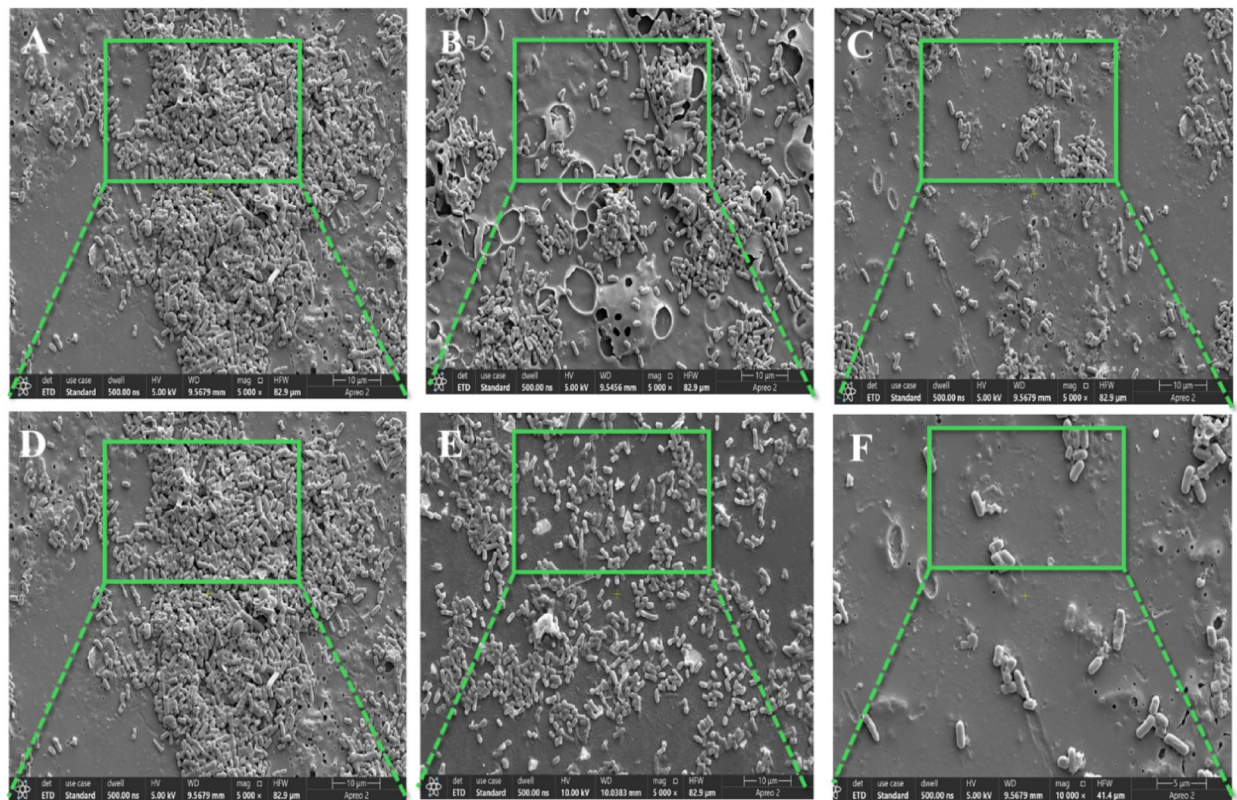


Fig. 5. SEM images of *E. coli* strain AR11E. Scanning Electron micrographs illustrating the biofilm formation by AR11E. A and D represent the control (untreated), B and C show biofilm treated with CO at sub-inhibitory and MIC respectively, while E and F display biofilm treated with CinO at sub-inhibitory and MIC. The green empty blocks marked the fields highlighting the potential degradation of bacteria by essential oils. * SEM: scanning electron microscope, MIC minimal inhibitory concentration, CO: Clove oil, CinO: Cinnamon oil.

Molecular characterization

Out of all the three SBPs only one i.e. AR22E contained *papC* gene which is one of the most potent virulent genes found to be reported in SBPs (Figure.9).

Discussion

In recent years poultry growth and production has increased in Pakistan, making it the 11th largest global producer of poultry. According to the report published on the situational analysis of Poultry Industry Amidst COVID 19 approximately 1.163 to 1.94 million tons of broiler chicken are currently being produced across more than 15,000 farms [55]. Unfortunately, the growth of hygienic raw food processing technology and practices does not parallel the production unlike many other developing countries⁵³. Moreover, the cause of concern beside the unhygienic practices is the rampant use of antibiotic as growth promoters and hygiene alternatives; and to make the matters worse there is non-existence of organized system to control the uncontrolled use of antimicrobials in the poultry industry^{54,55}. Neither is there any proper surveillance system to monitor antimicrobial drug resistance. Thus, it can be rationally hypothesized that the modern production practices of using antibiotics through feed as well as water either for disease control, prevention or as growth promoters can be responsible for the reported antibiotic resistance.

E. coli, one of the many causes of foodborne infection, is a facultative anaerobe from the Enterobacteriaceae family. It commonly prefers to reside in the gastrointestinal tracts of humans, poultry and animals; thus, it is part of both human and animal microflora and lives in symbiosis providing the vitamins needed by the hosts^{56,57}. Moreover, its isolates are considered as the hygiene practice indicators because of their adaptability and flexibility to grow both aerobically and anaerobically at 37 °C and to survive the harsher environments; as well. The survival of its isolates is also attributable to the presence of peritrichous flagella in some and others being non-motile^{58–60}. Besides being adhering to and surviving on either the biotic or abiotic surface through enrichment of its accessory virulence genes, it also spoils both the odor and color of the food by hydrolyzing proteins through its proteolytic enzymes. Varied existence and survival niches help *E. coli* to not only spread via contact with contaminated soil, water and food; oral-fecal route; and/or direct contact with manure and animals; rather also cause number of the internal, intestinal and urinary tract infections. Furthermore, animal and human fecal contamination of underground and surface water sources also contributes to disease and resistance transmission cycle in nature^{61,62}.

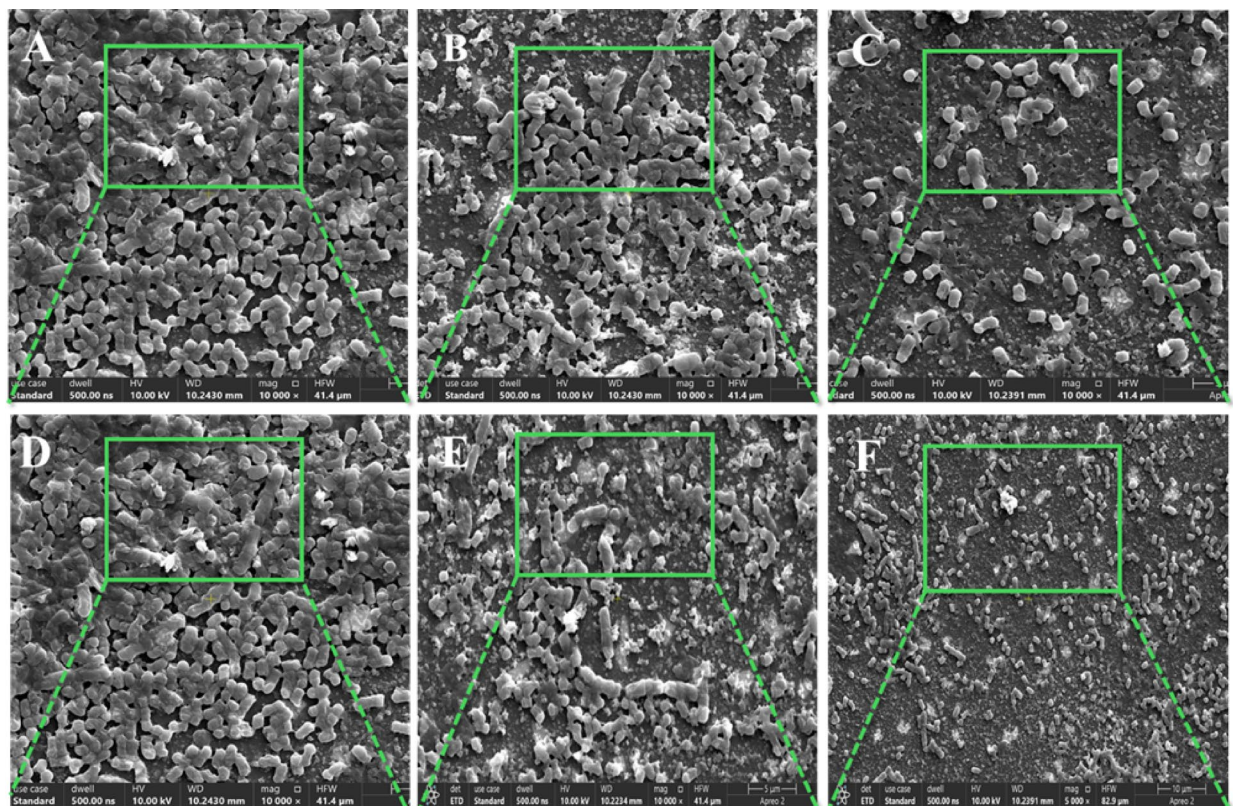


Fig. 6. SEM images of *E. coli* strain AR12E. Scanning Electron micrographs illustrating the biofilm formation by AR12E. A and D represent the control (untreated), B and C show biofilm treated with CO at sub-inhibitory and MIC respectively, while E and F display biofilm treated with CinO at sub-inhibitory and MIC. The green empty blocks marked the fields highlighting the potential degradation of bacteria by essential oils. * SEM: scanning electron microscope, MIC minimal inhibitory concentration, CO: Clove oil, CinO: Cinnamon oil.

Structural possessivity of flagella and being sessile allow *E. coli* to accumulate and undergo a complex and dynamic processes of forming biofilm^{63,64}. The biofilm is a complex collection of microorganisms in which they habitat synergistically; both the physical and chemical attributes of the biofilm microenvironment are different from the plankton cells^{65,66}. Biofilm formation is of grave concern in the food industry because it allows microorganisms like *E. coli* to adhere to the surfaces of the food and equipments⁶⁷. Biofilm formers not only tend to have survival edge, but rather they have the capability to evade the antimicrobials and disinfectants far more intelligently than their counterparts—the plankton cells^{68,69}.

The rising contamination and emergency of resistance to more than one commonly used antibiotics in *E. coli* is being reported in the food industry⁷⁰. In literature, the varying prevalence of *E. coli* in raw chicken meat has been reported. The highest being reported by Hussain et al. (India=78%) Jakaria et al. (Bangladesh=76.89%), Park et al. (Korea=75.9%), Rahman et al. (Bangladesh=63.5%), Rashid et al. (Egypt=40%), and Adeyanju et al. (Nigeria=47.2%)^{71–75}. In contrast the lower prevalence has been reported in Egypt (11.71%) and Iran (15.35%)^{76–78}. Whereas, in the current study, as compared to the aforementioned prevalence, only 49 (36.2%) samples out of 150 were morphologically identified as gram-negative rods and had the colonial characteristics of *E. coli*. The strong query coverage and identity of the sequenced 16SrRNA (Table 4) from three strains of *E. coli* (AR11E, AR12E and AR22E they are also SBPs) and strong agreement of the reconstructed cladograms with the reference strain of respective species further confirmed the identification of isolated strains. The aforementioned strains have also been allocated the accessioned numbers: AR11E=CP025739.1, AR12E=MN094135.1 and AR22E=MH608345.1 (Fig. 7A and B).

Interestingly, only seven samples out of 49 (i.e. 14.28%) were hemolysin producers and potentially pathogenic (Figure.1 A & B). The isolates were also screened for biofilm formation; since it is not only the part of their survival mechanism against the environment and the host rather it is also a pathogenic characteristic which enhances their virulence. 20 (40.8%) isolates were NBPs with OD = < 0.155, whereas the remaining 29 (59.2%) showed varying degree of biofilm producing ability: 9 SBPs (18.3%, OD = > 0.62), 6 MBPs (12.2%, OD = 0.31 to 0.62) and 14 WBPs (28.5%, OD = 0.155 to 0.31) (Figure.2). Comparatively, cited prevalence of SBPs in literature were 70.44%, 46%, 24.2% and 25.39% in Brazil, India, Turkey and China; respectively^{79–82}. Similarly, in a study conducted by Theeb Al-Marri et al. 57 isolates out of 86 were BPs and. 22 (25.6%) of them were SBPs⁸³. However, our study has reported a lower prevalence of SBPs. P-fimbrial usher protein (*papC*) is one of the virulence-associated adhesion factor genes (AFGs) reported in literature in the context of biofilm formation and has been concluded by Naves and group (2008) to have strong correlation with biofilm forming ability of the bacteria

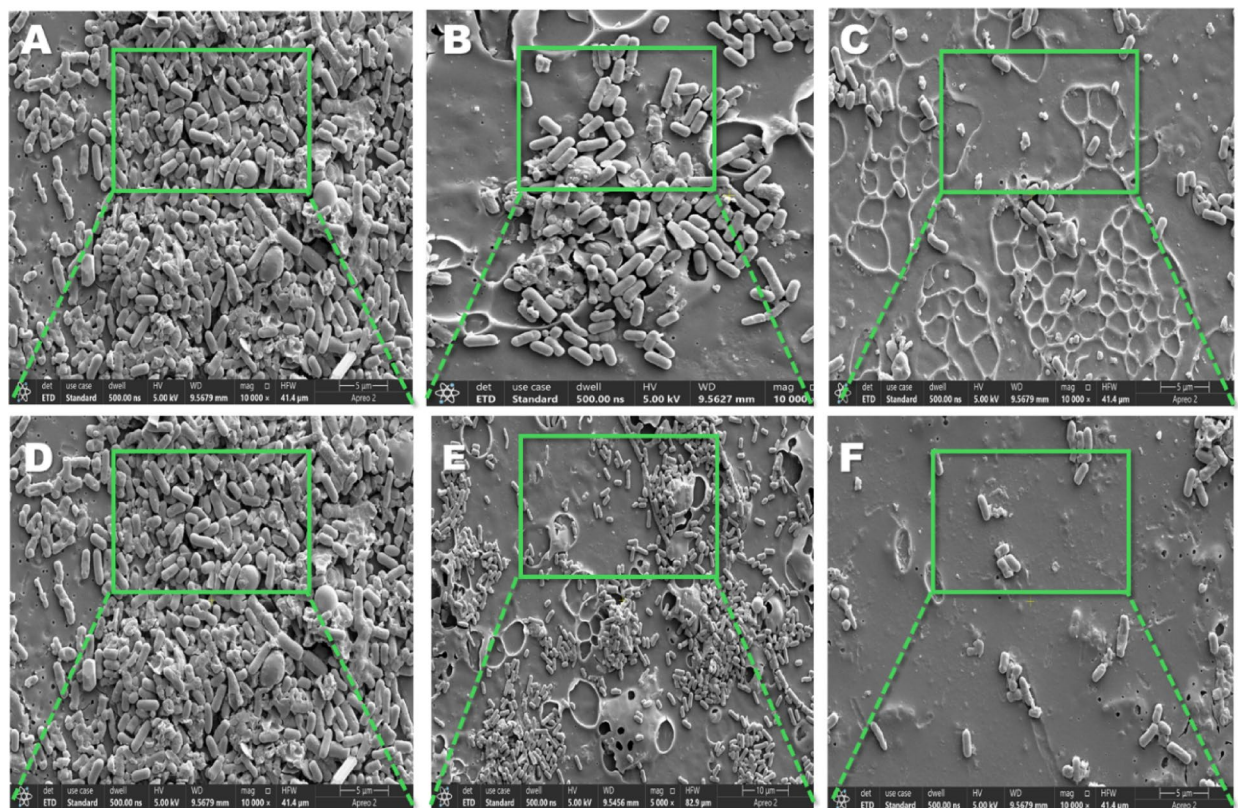


Fig. 7. SEM images of *E. coli* strain AR22E. Scanning Electron micrographs illustrating the biofilm formation by AR22E. A and D represent the control (untreated), B and C show biofilm treated with CO at sub-inhibitory and MIC respectively, while E and F display biofilm treated with CinO at sub-inhibitory and MIC. The green empty blocks marked the fields highlighting the potential degradation of bacteria by essential oils. * SEM: scanning electron microscope, MIC minimal inhibitory concentration, CO: Clove oil, CinO: Cinnamon oil.

and therefore, more common in strong biofilm producers⁸⁴. Specific fimbriae associated with pyelonephritis-associated pili (pap) is coded by *papC* gene. It was identified in only one of the SBP (AR22E) out of the three tested strains: AR12E, AR11E, AR22E (Figure.9). When compared to the reported percentage of *papC* (8.7% and 0%) our study showed a positive rate of 33%^{81,85}. This result is still inconclusive since all the SBPs were not probed for presence of *papC*. The budget constraints did not allow us to target more biofilm-associated adhesion genes like: *fimC*, which is also reported to be the most prevalent and its association with other genes like *tsh*, *papG* and *crl* is also the characteristic finding in the avian pathogenic strains of *E. coli*^{86,87}. Furthermore, associations of adhesion genes to reported mutations like *ompR* should also have been explored in order to evaluate the production of other adhesins and interaction of *E. coli* strains with both biotic and abiotic surfaces⁸⁸. Moreover, other virulence genes like the extended spectrum beta-lactamases enzymes (ESBLs) need to be probed; as well— for the identification of their putative association to biofilm production, in order to reach the more robust evidence-based conclusions.

The Kirby-Bauer disk diffusion method was employed to demonstrate antimicrobial susceptibility pattern of 49 isolates of *E. coli* against eight antibiotics. The results showed a higher prevalence of multidrug resistance (MDR) i.e. 90% (44 isolates) of them were resistant to at least three antibiotics, which agrees with the findings cited in the literature. It was observed in our study that 90% of the isolates were resistant to antibiotics commonly used in clinical practice. The susceptibility pattern showed 97.96%, 85.71%, 81.63% and 51.02% were resistant to cefoxitin, erythromycin, chloramphenicol and amoxicillin-clavulanate; and ciprofloxacin, tetracycline, and streptomycin; respectively. However, very interestingly 33 (67.35%) isolates of *E. coli* were sensitive to gentamicin. Our findings are more or less in agreement with what has been cited by Rahman et al., and Akond et al.^{89–91}. Similarly, in 2013 Tesfaheywet et al., demonstrated that *E. coli* isolated from broiler chicken were resistant to ampicillin (60%), streptomycin (78%) and tetracycline (90%). Our and Tesfaheywet et al.'s *E. coli* isolates showed a similar sensitivity pattern for gentamicin⁹². Higher prevalence of MDR *E. coli* have also been cited in the neighboring countries like Nepal (80%), Bangladesh (75.06%), Iran (64.91%), and India (60%)^{53,93–95}. Such high reported prevalence from our study and from various countries is alarming since it shows that resistant bugs are replacing the sensitive ones in an environment saturated with antibiotics. Interestingly, negative correlation has been reported in the literature which more or less agrees to statistically insignificant pairwise correlation for nine isolates of *E. coli* with strong biofilm production and antibiotic resistance ($r = < 0.50$) with the exception of erythromycin ($r = 0.59$) in our study (Table 2)⁹⁶.

In the era of antimicrobial resistance, the use of chemical preservatives/traditional antibiotics is not a wise choice to prevent and break the cycle of transmission/resistance among commensal and pathogenic

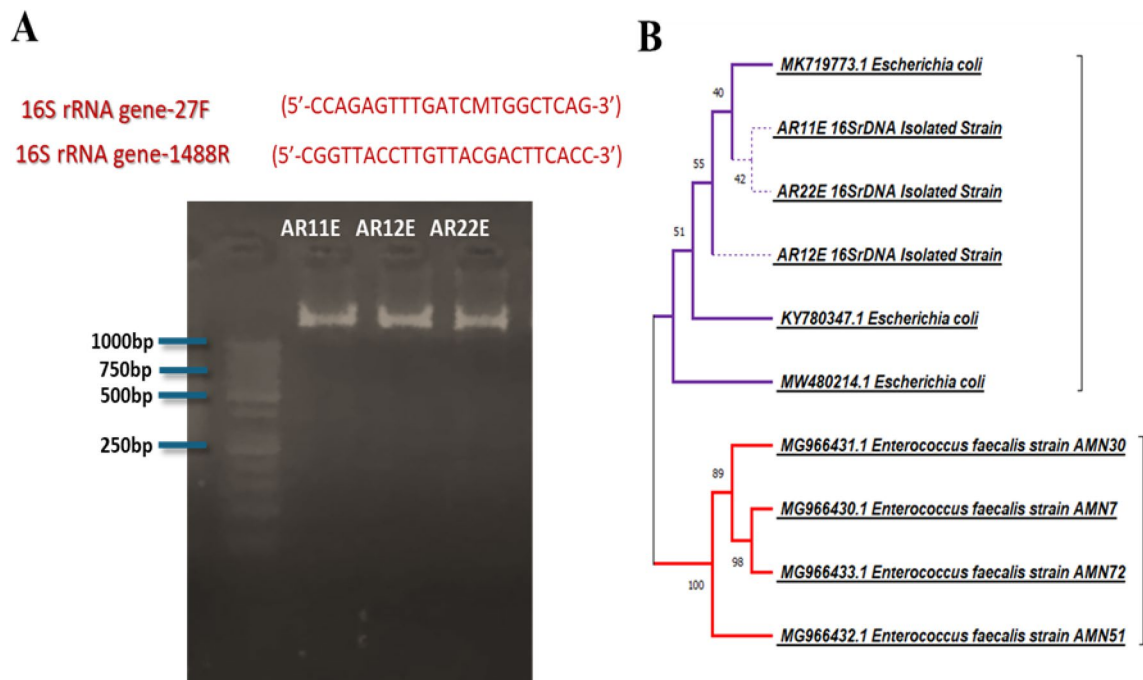


Fig. 8. PCR Amplification of 16SrRNA Gene. **(A)** Gel showing micrograph of extracted genomic DNA of 3 *Escherichia coli* strains. Amplified 16SrRNA band of ± 1.4 Kbp at 49 °C is appreciated in the graph. **(B)** Cladogram Reconstruction. The neighbor Joining tree was reconstructed based on 16SrRNA sequences of isolated *E. coli* strains; AR11E, AR12E and AR22E. The tree is rooted with 16SrRNA sequences of reference *E. coli* and *E. faecalis* strains. Subsequent cladogram nearly separated both genera by showing two distinct clades of *E. coli* (purple) and *E. faecalis* (red) revealing the genetic variability among strains. Bootstrap values (1000 replicates) are shown at the nodes.

<i>E. coli</i> strains	Best match after blast analysis	Query coverage	Query Identity	Accession number
AR22E	<i>Escherichia coli</i>	80%	93.71%	MH608345.1
AR11E	<i>Escherichia coli</i>	86%	98.78%	CP025739.1
AR12E	<i>Escherichia coli</i>	93%	97.39%	MN094135.1

Table 4. Blast Results of *E. coli* strains

microorganisms in food chain. Rather eco-friendly and novel means are required to break this vicious pattern and ensure the longevity of quality food products. Recently, many eco-friendly and sustainable strategies have been explored, which are also recommended as part of “One Health” approach. This approach is an integrated and unifying means of attempting to protect animals and prioritize public health. The application of essential oils is one such approach⁹⁷. They are composed of a varied number of biologically active compounds like phenyl propenes (eugenol and cinnamaldehyde) or phenolic monoterpenes (thymol and carvacrol). The richness of their composition allows them to have multiple biological actions along with synergistic interactions and makes them differ from the single isolated or synthetic molecules. Among the innumerable number of EOs with remarkable properties we chose clove and cinnamon to explore in our study. They are used both as spice and flavoring agents with potential medicinal benefits⁹⁸.

Clove EO has varied applications in both the health and food industry due to its putative bactericidal, antitumor-genic, antioxidant and analgesic activities^{99–101}. Besides, the aforementioned activities; it is also used both as natural food colorant and preservative^{102,103}. Furthermore, it is also employed as a wash treatment to increase the shelf-life and decrease the microbiological hazard in freshly cut vegetables. Thus, it is a sustainable and environment-friendly alternative to conventional chlorine-based disinfectants, sodium bicarbonate, and acetic acid^{104,105}. Its antibacterial property is attributable to eugenol, caryophyllene and humulene— eugenol, an aromatic extract, makes up to 60–90% of the total composition of oil^{31,106}. In 2021 Caballeo-Prado et al., reported a decrease in the Shiga toxin gene expression after the use of eugenol³¹. Cui *et al.*, cited in 2016 that clove oil at a concentration of 4 mg/mL not only reduced the viable *E. coli*’s in the biofilm on lettuce by 2.94 log CFU rather, the group also reported that 1 mg/mL of clove oil after 5 and 30 min reduced viable biofilm cells by 1.01 and 3.32 log CFU: respectively¹⁰⁷. Thus, concluding that viability of the biofilm cells is influenced by both the time of exposure and concentration of the clove oil. Similarly, Sharma et al. employed three concentrations of clove EO i.e. 1 wt%, 5 wt%, and 10 wt% on blended film of (poly(lactide)/poly (butylene adipate-co-terephthalate) designed

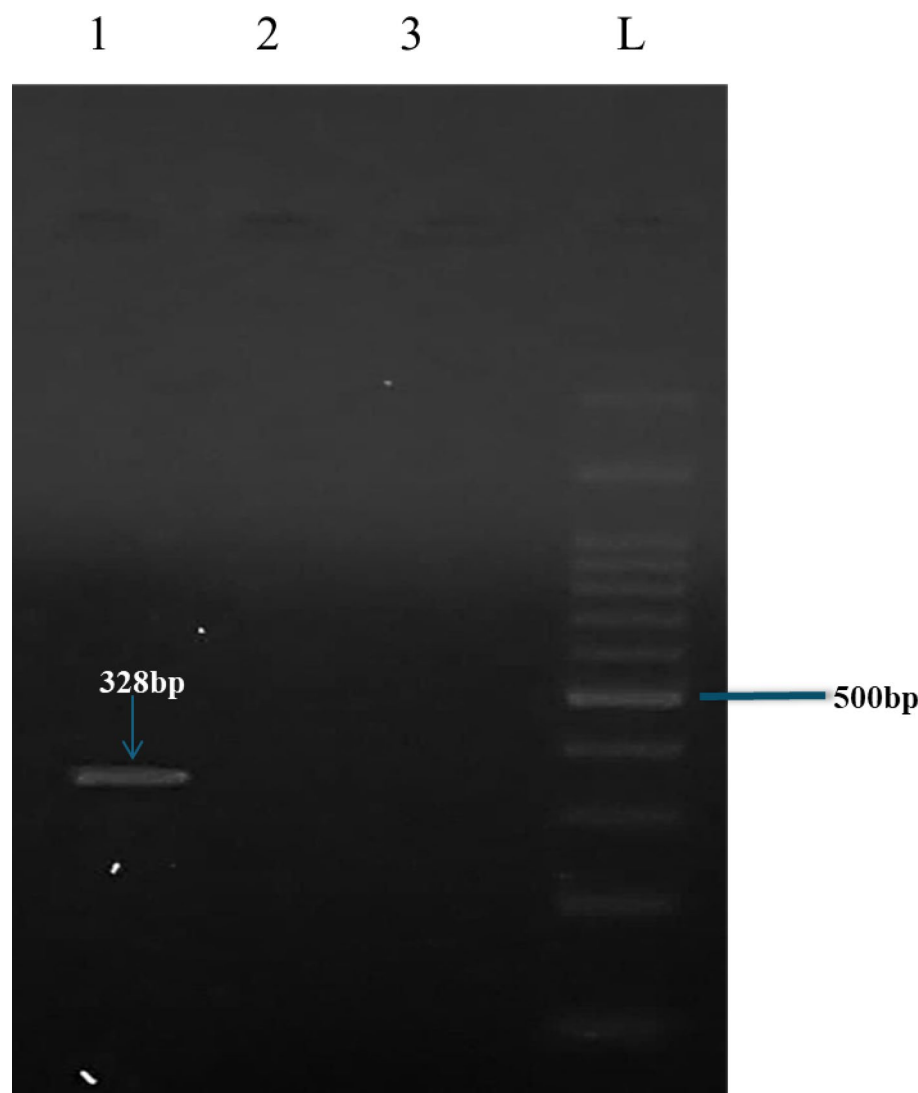


Fig. 9. PCR Amplification of *PapC* Gene. Agarose gel micrograph of *Escherichia coli* strains. Lane 1–3: test samples; AR11E, AR22E and AR12E. The *PapC* gene was detected in only one strain i.e. AR22E (lane 3). Lane (L) represents the DNA ladder.

for food packaging. The group used the blended film for a period of 24 h at three different concentrations to evaluate the antimicrobial activity of clove EO against *E. coli*. The antibacterial activity of film with 1 wt% of clove EO against *E. coli* lasted for 4 h whereas; the one with 5 wt% had prolonged activity of about 12 h and its effectiveness waned thereof. Very interestingly, *E. coli* growth was substantially reduced from initial count of 6.5 to 4.4 log CFU/mL when incubated with 10wt% blended film^{108–110}. Furthermore, the MIC and MBC of 0.037 mg/mL^{−1} and 1.064–4.36 mg mL^{−1}; respectively of clove EO for *E. coli* have been reported by de Almeida and group¹¹¹. In a nutshell both eugenol and clove EO have been cited in the literature to have confirm activity against pathogenic *E. coli*, *Pseudomonas aeruginosa*, *Streptococcus mutans*, *S. aureus* and *Proteus mirabilis*^{106,111}.

Belonging to the family Lauraceae and genus of *Cinnamomum*, 250 species of Cinnamon are reported in the literature. It is native to Australia, China and India and has widespread applications in medical, food and cosmetic industry due its antioxidant, anticarcinogenic and antimicrobial potential. Many of its activities are attributable to one of its active components; cinnamaldehyde (65–78%)^{112–114}. It scavenges reactive oxygen species through its phenolic content and also reduces the lipid peroxidation^{113,115}. According to de Almeida et al., Cinnamon stood out to be the most effective EO demonstrating lowest concentrations i.e. MIC of 0.052–0.178 mg/mL^{−1} and MBC of 0.058–1.084 mg/mL^{−1} against *E. coli*¹¹¹. Similarly, Yunbin Zhang et al., reported an MIC of cinnamon EO to be 1.0 mg/mL¹¹⁶. Viable cells in the *E. coli* biofilm in the carp fish flesh fingers were reduced by 2.58 log CFU when treated with cinnamon EO by disrupting the regulation of battery of virulence genes: *qseC*, *stx2*, *luxS*, *qseB* and *hfq*^{117,118}. Relevant to the above study, Sheng et al., observed that adding 0.75 MIC of cinnamon EO to the culture medium not only down regulated all the aforementioned genes rather, reduced both the expression and production of the Shiga toxin type 2 (*stx2*) gene and the protein (Stx2); respectively¹¹⁹. Similar findings were also cited by Yuan et al.¹²⁰. Both Wen et al., and Raeisi et al., incorporated the EO into polylactic acid

(PLA) nanofibers and sodium alginate coating; respectively and found that there was significant reduction in the growth of bacteria and the shelf life of chicken meat increased^{121,122}.

The MIC values of both CO and CinO against *E. coli* reported by our study are in agreement with the aforementioned evidence cited here from the literature. A concentration of 62.5 µL/mL of CinO inhibited approximately 40% of isolates whereas, 250 to 500 µL/mL of CO was required to show similar inhibition pattern (Figure.4). Notably, one of the isolates (AR11E) with strong biofilm forming ability had considerable resilience to both CinO and CO and was inhibited at 500 and 1000 µL/mL; respectively. The lower MIC of CinO is required to effectively inhibit the isolates of *E. coli* as compared to CO, which is in agreement with results cited by Raeisi et al., and Adesiji et al.^{121,123}; however, our results are in disagreement to what has been cited by Asha et al., it was found that high antibacterial activity against *E. coli* was observed with CO rather than CinO¹²⁴.

The anti-biofilm effect of both EOs on the morphology and structure of SBPs: AR11E, AR12E and AR22E were evaluated through SEM. (Figure.5). The scanning microscopy highlighted the potential degradation of the bacterial cells. *The credence to the biofilm activity could have been added by performing anti-biofilm inhibition assay; as well. Furthermore, we did not explore the synergism between both the oils as it has been recently observed by de Almeida et al., that better antimicrobial activity occurs when clove and cinnamon EO are explored together* [116]. The gram-positive bacteria are reported to be more susceptible to CinO and CO¹²⁵; however, our results showed considerable inhibitory activity of both the oils against the *E. coli* isolates; as well.

Conclusions and future perspectives

Comparatively, our reported prevalence of *E. coli* and SBPs in retail chicken meat is not very high as to what has been cited in literature, *probably due to the smaller number of samples*. However, the majority (90%) of the isolates were resistant to more than three antibiotics indicating that not only the fecal contamination and unhygienic practices at slaughterhouse, processing units and retail wet shops are widespread rather there may be unreported judicious use of antimicrobials; as well. *The power of the study could be increased by exploring the larger number of samples*. Moreover, it is important to not only keep a tab on the prevalence of these commensal and pathogenic microorganisms rather track the occurrence of antimicrobial resistance in food commodities like retail chicken meat since they are an exposure site close to the consumer.

Use of essential oils like CinO and CO in poultry meat and meat products can be a good strategy since they have proven efficacy against the chicken meat contaminated with pathogenic *E. coli*. However, studies on food systems (i.e. chicken meat) exploring the safety profile and organoleptic effects are required to support the in-vitro evidence cited in herein and literature. Interestingly, these spices are used as taste enhancers as well in the final meat product therefore, their application in the raw meat is particularly well suited as compared to other foods. Moreover, the biological active components found in these essential oils are safe for human consumption since they have short half-life and are eliminated in due course of time through the kidneys¹²⁶.

The bactericidal and bacteriostatic activities of EOs are dependent on the composition of both the food matrix and the oil itself along with the processing and application strategies¹¹⁸. In future the antimicrobial efficacy of both CinO and CO observed in our study can be augmented by associating it with other natural substances—like utilizing the concept of combining three plant-derived phenolic principles like thymol (thyme), carvacrol (thyme and oregano) and eugenol (clove and cinnamon)^{127,128}. This principle seems to show greater antimicrobial efficacy against *E. coli* as compared to the individual phenolic compound in single EO. It also tends to decrease the amount of oil used. Interestingly, CinO is also approved by the Food and Drug Administration as phytochemical feed additive (PFA) in poultry feed since it improves the gut microbiota of poultry by promoting the growth of healthy commensal bacteria^{129–131}.

Data availability

The 16S rRNA acquired gene sequences were submitted in the NCBI database, and the accession numbers are mentioned in Table 4. The data generated in current study is available in NCBI database with accession numbers MH608345.1, CP025739.1 and MN094135.1.

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References

- World Bank Open Data [Internet]. [cited 2025 Feb 6]. World Bank Open Data. Available from: <https://data.worldbank.org>
- IMF [Internet]. [cited 2025 Feb 6]. World Economic Outlook Database, October 2023. Available from: <https://www.imf.org/en/Publications/WEO/weo-database/2023/October>
- OECD [Internet]. [cited 2025 Feb 6]. Meat consumption. Available from: <https://www.oecd.org/en/data/indicators/meat-consumption.html>
- Statistics [Internet]. [cited 2025 Feb 6]. Agricultural production statistics 2010–2023. Available from: <https://www.fao.org/statistics/highlights-archive/highlights-detail/agricultural-production-statistics-2010-2023/en>
- Hedman, H. D., Vasco, K. A. & Zhang, L. A review of antimicrobial resistance in poultry farming within Low-Resource settings. *Anim. Open. Access. J. MDPI*. **10** (8), 1264 (2020).
- Odeyemi, O. A., Alegbeleye, O. O., Strateva, M. & Stratev, D. Understanding spoilage microbial community and spoilage mechanisms in foods of animal origin. *Compr. Rev. Food Sci. Food Saf.* **19** (2), 311–331 (2020).
- Darwish, W. S. et al. Chicken giblets and wastewater samples as possible sources of methicillin-resistant *Staphylococcus aureus*: prevalence, enterotoxin production, and antibiotic susceptibility. *J. Food Saf.* **38** (4), e12478 (2018).
- Darwish, W. S., El-Ghareeb, W. R., Alsayeqh, A. F. & Morshdy, A. E. M. A. Chapter 4 - Foodborne intoxications and toxicoinfections in the Middle East. In: Savvaidis IN, Osaili TM, editors. *Food Safety in the Middle East* [Internet]. Academic Press; [cited 2025 Feb 9]. pp. 109–41. (2022). Available from: <https://www.sciencedirect.com/science/article/pii/B9780128224175000015>
- Mahmoud, A. F. A., Mohamed, E. A. A., Hussein, M. A. M. & Darwish, W. Biological hazards associated with chicken meat: A review. *J. Adv. Vet. Res.* **13** (8), 1717–1720 (2023).

10. Morshdy, A. E. M. A., Mehrez, S. M. I., Tharwat, A. E., Abdallah, K. M. E. & Darwish, W. A review on the microbial surface contaminants of the animal carcasses. *J. Adv. Vet. Res.* **13** (6), 1248–1251 (2023).
11. Estimates of Foodborne Illness in the United States | Estimates of Foodborne Illness | CDC [Internet]. 2019 [cited 2025 Feb 6]. Available from: <https://www.cdc.gov/foodborneburden/index.html>
12. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2017 | EFSA [Internet]. 2018 [cited 2025 Feb 6]. Available from: <https://www.efsa.europa.eu/en/efsajournal/pub/5500>
13. Kirk, M., Ford, L., Glass, K. & Hall, G. Foodborne illness, australia, circa 2000 and circa 2010. *Emerg. Infect. Dis.* **20** (11), 1857–1864 (2014).
14. Bai, J. et al. Antibacterial activity and mechanism of clove essential oil against foodborne pathogens *LWT*, **173**, 114249 (2023).
15. Darwish, W. & Thompson, L. In. Soil, water, and air: potential contributions of inorganic and organic chemicals. In present Knowledge in Food Safety pp. 26–43. (2023).
16. Antimicrobial resistance [Internet]. [cited 2025 Feb 7]. Available from: <https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance>
17. Darwish, W. S. & Thompson, L. A. Chapter 2 - Soil, water, and air: potential contributions of inorganic and organic chemicals. In: Knowles ME, Anelich LE, Boobis AR, Popping B, editors. Present Knowledge in Food Safety [Internet]. Academic Press; [cited 2025 Feb 8]. pp. 26–43. (2023). Available from: <https://www.sciencedirect.com/science/article/pii/B9780128194706000378>
18. Luna-Guevara, J. J., Arenas-Hernandez, M. M. P., de la Martínez, C., Silva, J. L. & Luna-Guevara, M. L. The role of pathogenic *E. coli* in fresh vegetables: behavior, contamination factors, and preventive measures. *Int. J. Microbiol.* **2019**, 2894328 (2019).
19. Hassan Ali, N., Farooqui, A., Khan, A., Khan, A. Y. & Kazmi, S. U. Microbial contamination of Raw meat and its environment in retail shops in karachi, Pakistan. *J. Infect. Dev. Ctries.* **4** (6), 382–388 (2010).
20. Hu, W., Li, C., Dai, J., Cui, H. & Lin, L. Antibacterial activity and mechanism of litsea Cubeba essential oil against methicillin-resistant *Staphylococcus aureus* (MRSA). *Ind. Crops Prod.* **130**, 34–41 (2019).
21. Wang, F. et al. *Dodartia orientalis* L. essential oil exerts antibacterial activity by mechanisms of disrupting cell structure and resisting biofilm. *Ind. Crops Prod.* **109**, 358–366 (2017).
22. Akhtar, N. & Ihsan-ul-Haq, Mirza, B. Phytochemical analysis and comprehensive evaluation of antimicrobial and antioxidant properties of 61 medicinal plant species. *Arab. J. Chem.* **11** (8), 1223–1235 (2018).
23. Srey, S., Jahid, I. K. & Ha, S. D. Biofilm formation in food industries: A food safety concern. *Food Control.* **31** (2), 572–585 (2013).
24. Yuan, L. H., Hu, Q. P. & Xu, J. G. Antibacterial mechanism of the Ethyl acetate extracts from naked oat again *Bacillus subtilis*. *Aceh Int. J. Sci. Technol.* **4** (1), 20–25 (2015).
25. Cui, H., Zhang, C., Li, C. & Lin, L. Antimicrobial mechanism of clove oil on *Listeria monocytogenes*. *Food Control.* **94**, 140–146 (2018).
26. Cui, H., Zhang, X., Zhou, H., Zhao, C. & Lin, L. Antimicrobial activity and mechanisms of salvia sclarea essential oil. *Bot. Stud.* **56** (1), 16 (2015).
27. Cui, H., Zhao, C. & Lin, L. The specific antibacterial activity of liposome-encapsulated Clove oil and its application in Tofu. *Food Control.* **56**, 128–134 (2015).
28. Essential Oils: Oils of Nature. - Google Books [Internet]. [cited 2025 Feb 8]. Available from: https://books.google.com.pk/book?hl=en&lr=&id=B3L8DwAAQBAJ&oi=fnd&pg=PA147&dq=+Comparative+analysis+of+the+chemical+composition,+antimicrobial+and+antioxidant+activity+of+essential+oils+of+spices+used+in+the+food+industry+in+Brazil.+In+Essential+Oils+Of+Nature.+IntechOpen.&ots=u1WSRoqRyI&sig=VWY2r88BSmZuzNKF59_AGtvpV0&redir_esc=y#v=onepage&q=Comparative%20analysis%20of%20the%20chemical%20composition%2C%20antimicrobial%20and%20antioxidant%20activity%20of%20essential%20oils%20of%20spices%20used%20in%20the%20food%20industry%20in%20Brazil.%20In%20Essential%20Oil+s-Oils%20of%20Nature.%20IntechOpen.&f=false
29. Latifah-Munirah, B., Himratul-Aznita, W. H. & Mohd Zain, N. Eugenol, an essential oil of clove, causes disruption to the cell wall of *Candida albicans* (ATCC 14053). *Front. Life Sci.* **8** (3), 231–240 (2015).
30. Rajkowska, K., Otlewska, A., Kunicka-Styczyńska, A. & Krajewska, A. *Candida albicans* impairments induced by peppermint and clove oils at Sub-Inhibitory concentrations. *Int. J. Mol. Sci.* **18** (6), 1307 (2017).
31. Wongsawan, K., Chaisri, W., Tangtrongsup, S. & Mektrirar, R. Bactericidal effect of clove oil against Multidrug-Resistant *Streptococcus suis* isolated from human patients and slaughtered pigs. *Pathogens* **9** (1), 14 (2019).
32. Burt, S. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int. J. Food Microbiol.* **94** (3), 223–253 (2004).
33. Kuhn, D. et al. Antibiofilm activity of the essential oil of *Campomanesia aurea* O. Berg against microorganisms causing food borne diseases. *LWT* **108**, 247–252 (2019).
34. Tian, J. et al. Regional variation in components and antioxidant and antifungal activities of *Perilla frutescens* essential oils in China. *Ind. Crops Prod.* **59**, 69–79 (2014).
35. Alirezalu, K. et al. Phytochemical constituents, advanced extraction technologies and techno-functional properties of selected mediterranean plants for use in meat products. A comprehensive review. *Trends Food Sci. Technol.* **100**, 292–306 (2020).
36. da Silva, B. D., Bernardes, P. C., Pinheiro, P. F., Fantuzzi, E. & Roberto, C. D. Chemical composition, extraction sources and action mechanisms of essential oils: natural preservative and limitations of use in meat products. *Meat Sci.* **176**, 108463 (2021).
37. Pateiro, M. et al. Essential oils as natural additives to prevent oxidation reactions in meat and meat products: A review. *Food Res. Int.* **113**, 156–166 (2018).
38. Pateiro, M. et al. Application of essential oils as antimicrobial agents against spoilage and pathogenic microorganisms in meat products. *Int. J. Food Microbiol.* **337**, 108966 (2021).
39. İNanç, A. & Mustafa, A. S. Tavuk Etlerinden İzole edilen *E. Coli* O157:H7 antibiyotik direnci. Kahramanmaraş Sütçü İmam Üniversitesi Doğa Bilim Derg [Internet]. 2018 Aug 15 [cited 2025 Feb 7];21(1). Available from: <https://dergipark.org.tr/en/doi/https://doi.org/10.18016/ksudobil.289192>
40. Hussein, H. S., Bollinger, L. M. & Hall, M. R. Growth and enrichment medium for detection and isolation of Shiga Toxin-Producing *Escherichia coli* in cattle feces. *J. Food Prot.* **71** (5), 927–933 (2008).
41. Stulik, L. et al. α -Hemolysin activity of Methicillin-Susceptible *Staphylococcus aureus* predicts Ventilator-associated pneumonia. *Am. J. Respir Crit. Care Med.* **190** (10), 1139–1148 (2014).
42. Hassan, A. et al. Evaluation of different detection methods of biofilm formation in the clinical isolates. *Braz J. Infect. Dis.* **15** (4), 305–311 (2011).
43. Taj, Y., Essa, F., Aziz, F. & Kazmi, S. U. Study on biofilm-forming properties of clinical isolates of *Staphylococcus aureus*. *J. Infect. Dev. Ctries.* **6** (05), 403–409 (2012).
44. Coffey, B. M. & Anderson, G. G. Biofilm formation in the 96-well microtiter plate. *Methods Mol. Biol. Clifton NJ.* **1149**, 631–641 (2014).
45. De Jesus, R. & Dedes, G. Data on quantitation of *Bacillus cereus sensu lato* biofilms by microtiter plate biofilm formation assay. *Data Brief.* **28**, 104951 (2020).
46. Kosikowska, U. et al. Prevalence of susceptibility patterns of opportunistic bacteria in line with CLSI or EUCAST among *Haemophilus parainfluenzae* isolated from respiratory microbiota. *Sci. Rep.* **10** (1), 11512 (2020).
47. Wheat, P. F. History and development of antimicrobial susceptibility testing methodology. *J. Antimicrob. Chemother.* **48** (suppl_1), 1–4 (2001).

48. Weerakkody, V., El-Haddadeh, R., Al-Sobhi, F., Shareef, M. A. & Dwivedi, Y. K. Examining the influence of intermediaries in facilitating e-government adoption: an empirical investigation. *Int. J. Inf. Manag.* **33** (5), 716–725 (2013).
49. Han, Z. et al. MICS-Asia II: model intercomparison and evaluation of Ozone and relevant species. *Atmos. Environ.* **42** (15), 3491–3509 (2008).
50. Barabadi, H. et al. Green synthesis, characterization, antibacterial and biofilm inhibitory activity of silver nanoparticles compared to commercial silver nanoparticles. *Inorg. Chem. Commun.* **129**, 108647 (2021).
51. Ganderton, L., Chawla, J., Winters, C., Wimpenny, J. & Stickler, D. Scanning electron microscopy of bacterial biofilms on indwelling bladder catheters. *Eur. J. Clin. Microbiol. Infect. Dis.* **11** (9), 789–796 (1992).
52. Clifford, R. J. et al. Detection of bacterial 16S rRNA and identification of four clinically important bacteria by Real-Time PCR. *PLoS ONE* **7** (11), e48558 (2012).
53. Rahman, M. M. et al. Isolation and molecular characterization of multidrug-resistant *Escherichia coli* from chicken meat. *Sci. Rep.* **10**, 21999 (2020).
54. Moreno, A., Bello, H., Guggiana, D., Domínguez, M. & González, G. Extended-spectrum β -lactamases belonging to CTX-M group produced by *Escherichia coli* strains isolated from companion animals treated with Enrofloxacin. *Vet. Microbiol.* **129** (1), 203–208 (2008).
55. Okeke, I. N., Lamikanra, A. & Edelman, R. Socioeconomic and behavioral factors leading to acquired bacterial resistance to antibiotics in developing countries. *Emerg. Infect. Dis.* **5** (1), 18–27 (1999).
56. Roque-Borda, C. A. 1, Bento da Silva P 2, Rodrigues 2 MC, Azevedo 2 RB, Filippo 3 LD, Duarte JL 3, et al. Challenge in the Discovery of New Drugs: Antimicrobial Peptides against WHO-List of Critical and High-Priority Bacteria. :773. (2021).
57. Zhang, W. et al. Prevalence of colistin resistance gene *mcr-1* in *Escherichia coli* isolated from chickens in central china, 2014 to 2019. *J. Glob. Antimicrob. Resist.* **29**, 241–246 (2022).
58. Heredia, N. & García, S. Animals as sources of food-borne pathogens: A review. *Anim. Nutr.* **4** (3), 250–255 (2018).
59. Sadeghzadeh, R., Esfandiari, Z., Khaneghah, A. M. & Rostami, M. A review of challenges and solutions of biofilm formation of *Escherichia coli*: conventional and novel methods of prevention and control. *Food Bioprocess. Technol.* **17** (9), 2583–2618 (2024).
60. Sharma, G. et al. *Escherichia coli* biofilm: development and therapeutic strategies. *J. Appl. Microbiol.* **121** (2), 309–319 (2016).
61. Probert, W. S., Miller, G. M. & Ledin, K. E. Contaminated stream water as source for *Escherichia coli* O157 illness in children. *Emerg. Infect. Dis.* **23** (7), 1216–1218 (2017).
62. Sváb, D. et al. Virulence Gene, and Prophage Arrays of Bovine and Human Shiga Toxigenic and Enteropathogenic *Escherichia coli* Strains Isolated in Hungary. *Front. Microbiol.* [Internet]. Jul 5 [cited 2025 Feb 7];13. Available from: <https://www.frontiersin.org/journals/microbiology/articles/> (2022). <https://doi.org/10.3389/fmicb.2022.896296/full>
63. de Pontes, J. T. C., Toledo Borges, A. B., Roque-Borda, C. A. & Pavan, F. R. Antimicrobial peptides as an alternative for the eradication of bacterial biofilms of Multi-Drug resistant bacteria. *Pharmaceutics* **14** (3), 642 (2022).
64. Zhang, B. et al. Antibacterial activity and mechanism of slightly acidic electrolyzed water (SAEW) combined with ultraviolet light against *Staphylococcus aureus*. *LWT* **182**, 114746 (2023).
65. Bridier, A. et al. Biofilm-associated persistence of food-borne pathogens. *Food Microbiol.* **45**, 167–178 (2015).
66. Kim, U., Kim, J. H. & Oh, S. W. Review of multi-species biofilm formation from foodborne pathogens: multi-species biofilms and removal methodology. *Crit. Rev. Food Sci. Nutr.* **62** (21), 5783–5793 (2022).
67. Almasoud, A. et al. Inhibitory effects of lactic and malic organic acids on autoinducer type 2 (AI-2) quorum sensing of *Escherichia coli* O157:H7 and *Salmonella typhimurium*. *LWT - Food Sci. Technol.* **66**, 560–564 (2016).
68. Vidács, A., Kerekes, E. B., Takó, M., Vágvolgyi, C. & Krisch, J. Eradication of multiple-species biofilms from food industrial and domestic surfaces using essential oils. *Food Sci. Technol. Int.* **30** (4), 361–369 (2024).
69. Yuan, L., Sadiq, F. A., Wang, N., Yang, Z. & He, G. Recent advances in Understanding the control of disinfectant-resistant biofilms by hurdle technology in the food industry. *Crit. Rev. Food Sci. Nutr.* **61** (22), 3876–3891 (2021).
70. Mølbak, K. Spread of resistant bacteria and resistance genes from animals to Humans – The public health consequences. *J. Vet. Med. Ser. B* **51** (8–9), 364–369 (2004).
71. Adeyanju, G. T. & Ishola, O. *Salmonella* and *Escherichia coli* contamination of poultry meat from a processing plant and retail markets in ibadan, Oyo state, Nigeria. *SpringerPlus* **3**, 139 (2014).
72. Hussain, A. et al. Risk of transmission of antimicrobial resistant *Escherichia coli* from commercial broiler and Free-Range retail chicken in India. *Front. Microbiol.* **8**, 2120 (2017).
73. Jakaria, A. T. M., Islam, M. A. & Khatun, M. M. Prevalence, characteristics and antibiogram profiles of *Escherichia coli* isolated from apparently healthy chickens in mymensingh, Bangladesh. *Microbes Health.* **1** (1), 27–29 (2012).
74. Park, H. et al. Antibiotic resistance and virulence potentials of Shiga Toxin-Producing *Escherichia coli* isolates from Raw meats of slaughterhouses and retail markets in Korea *Journal of microbiology and biotechnology*, **25**(9), 1460–1466 (2015).
75. Rashid, M., Kotwal, S., Malik, M. & Singh, M. Prevalence, genetic profile of virulence determinants and multidrug resistance of *Escherichia coli* isolates from foods of animal origin. *Vet. World.* **6** (3), 139 (2013).
76. Moawad, A. A. et al. Occurrence of *Salmonella enterica* and *Escherichia coli* in Raw chicken and beef meat in Northern Egypt and dissemination of their antibiotic resistance markers. *Gut Pathog.* **9**, 57 (2017).
77. Ranjbar, R., Sheikhshahrokh, A. & Jonaidi Jafari, N. Shiga (vero) toxin producing *Escherichia coli* in various types of food stuffs; virulence factors, O-serogroups and antimicrobial resistance properties. *J. Food Saf.* **37** (2), e12312 (2017).
78. Younis, G. A., Elkenany, R. M., Fouda, M. A. & Mostafa, N. F. Virulence and extended-spectrum β -lactamase encoding genes in *Escherichia coli* recovered from chicken meat intended for hospitalized human consumption. *Vet. World.* **10** (10), 1281–1285 (2017).
79. Benameur, Q. et al. Virulence, antimicrobial resistance and biofilm production of *Escherichia coli* isolates from healthy broiler chickens in Western Algeria. *Antibiotics* **10** (10), 1157 (2021).
80. Crecencio, R. B. et al. Antimicrobial susceptibility, biofilm formation and genetic profiles of *Escherichia coli* isolated from retail chicken meat. *Infect. Genet. Evol.* **84**, 104355 (2020).
81. Dishan, A. et al. Biofilm formation, antibiotic resistance and genotyping of Shiga toxin-producing *Escherichia coli* isolated from retail chicken meats. *Br. Poult. Sci.* **64** (1), 63–73 (2023).
82. Wang, Y. et al. Isolation, phylogenetic group, drug resistance, biofilm formation, and adherence genes of *Escherichia coli* from poultry in central China. *Poult. Sci.* **95** (12), 2895–2901 (2016).
83. Al-Marri, T., Al-Marri, A., Al-Zanbaqi, R., Ajmi, A. A. & Fayeze, M. Multidrug resistance, biofilm formation, and virulence genes of *Escherichia coli* from backyard poultry farms. *Vet. World.* **14** (11), 2869–2877 (2021).
84. Naves, P. et al. Measurement of biofilm formation by clinical isolates of *Escherichia coli* is method-dependent. *J. Appl. Microbiol.* **105** (2), 585–590 (2008).
85. Barilli, E., Vismarra, A., Frascolla, V., Rega, M. & Bacci, C. *Escherichia coli* strains isolated from retail meat products: evaluation of biofilm formation ability, antibiotic resistance, and phylogenetic group analysis. *J. Food Prot.* **83** (2), 233–240 (2020).
86. Janben, T. et al. Virulence-associated genes in avian pathogenic *Escherichia coli* (APEC) isolated from internal organs of poultry having died from colibacillosis. *Int. J. Med. Microbiol. IJMM.* **291** (5), 371–378 (2001).
87. Mellata, M. et al. Role of avian pathogenic *Escherichia coli* virulence factors in bacterial interaction with chicken heterophils and macrophages. *Infect. Immun.* **71** (1), 494–503 (2003).
88. Liu, H., Wang, L., Xie, Z. & Shen, P. Involvement of DNA in biofilm formation II: from bacterial adhesion to biofilm formation. *Wuhan Univ. J. Nat. Sci.* **17** (2), 162–168 (2012).

89. MR., BMR., BR. Antibigram and Plasmid Profile Analysis of Isolated *Escherichia coli* from Broiler and Layer. *Res J Microbiol.* 2008 Feb 1;3(2):82–90.
90. Akond, M. A., Hassan, S. M. R., Alam, S. & Shirin, M. Antibiotic resistance of *Escherichia coli* isolated from poultry and poultry environment of Bangladesh. *Am. J. Environ. Sci.* 5 (1), 47–52 (2009).
91. Lee, C. W. et al. Characterization of highly pathogenic H5N1 avian influenza A viruses isolated from South Korea. *J. Virol.* 79 (6), 3692–3702 (2005).
92. Bedada, T. Z., ANTIMICROBIAL RESISTANT PATTERN OF FECAL *ESCHERICHIA COLI* IN SELECTED BROILER, B., FARMS OF EASTERN HARARGE ZONE & Int, E. T. H. I. O. P. I. A. *J Appl Biol P* [Internet]. 2013 [cited 2025 Feb 8]; Available from: <https://www.semanticscholar.org/paper/ANTIMICROBIAL-RESISTANT-PATTERN-OF-FECAL-COLI-IN-OF-Bedada/b4bf8d62503e2a78310243a272008308a4ce8ed>
93. Brower, C. H. et al. The prevalence of Extended-Spectrum Beta-Lactamase-Producing Multidrug-Resistant *Escherichia coli* in poultry chickens and variation according to farming practices in punjab, India. *Environ. Health Perspect.* 125 (7), 077015 (2017).
94. Momtaz, H., Rahimi, E. & Moshkelani, S. Molecular detection of antimicrobial resistance genes in *E. coli* isolated from slaughtered commercial chickens in Iran. *Veterinárni Medicina.* 57 (4), 193–197 (2012).
95. Shrestha, A. et al. Multi-drug resistance and extended spectrum beta lactamase producing gram negative bacteria from chicken meat in Bharatpur metropolitan, Nepal. *BMC Res. Notes.* 10 (1), 574 (2017).
96. Alshaikh, S. A., El-Banna, T., Sonbol, F. & Farghali, M. H. Correlation between antimicrobial resistance, biofilm formation, and virulence determinants in uropathogenic *Escherichia coli* from Egyptian hospital. *Ann. Clin. Microbiol. Antimicrob.* 23 (1), 20 (2024).
97. Gonelimali, F. D. et al. Antimicrobial Properties and Mechanism of Action of Some Plant Extracts Against Food Pathogens and Spoilage Microorganisms. *Front Microbiol* [Internet]. 2018 Jul 24 [cited 2025 Feb 8];9. Available from: <https://www.frontiersin.org/journals/microbiology/articles/https://doi.org/10.3389/fmicb.2018.01639/full>
98. Ogwaro, B., O'Gara, E. A., Hill, D. J. & Gibson, H. The effect of combined clove and cinnamon extracts on growth and survival of *Escherichia fergusonii* and *Salmonella typhimurium* in milk pre and post fermentation. 5 [Internet]. 2022 Mar 23 [cited 2025 Feb 8]; Available from: <https://wlv.openrepository.com/handle/2436/624666>
99. Bai, J. et al. Antibacterial activity and mechanism of clove essential oil against foodborne pathogens. *LWT* 173, 114249 (2023).
100. Das, M., Roy, S., Guha, C., Saha, A. K. & Singh, M. In vitro evaluation of antioxidant and antibacterial properties of supercritical CO₂ extracted essential oil from clove bud (*Syzygium aromaticum*). *J. Plant. Biochem. Biotechnol.* 30 (2), 387–391 (2021).
101. Kheawfu, K., Pikulkaew, S., Rades, T., Müllertz, A. & Okonogi, S. Development and characterization of clove oil nanoemulsions and self-microemulsifying drug delivery systems. *J. Drug Deliv Sci. Technol.* 46, 330–338 (2018).
102. Ju, J. et al. A novel method to prolong bread shelf life: sachets containing essential oils components. *LWT* 131, 109744 (2020).
103. Kumar Pandey, V. et al. A comprehensive review on clove (*Caryophyllus aromaticus* L.) essential oil and its significance in the formulation of edible coatings for potential food applications. *Front Nutr* [Internet]. Sep 15 [cited 2025 Feb 8];9. Available from: <https://www.frontiersin.org/journals/nutrition/articles/https://doi.org/10.3389/fnut.2022.987674/full>
104. Ju, J. et al. Inhibitory effects of cinnamon and clove essential oils on mold growth on baked foods. *Food Chem.* 240, 850–855 (2018).
105. Park, J. B., Kang, J. H. & Song, K. B. Clove bud essential oil emulsion containing benzethonium chloride inactivates *Salmonella typhimurium* and *Listeria monocytogenes* on fresh-cut Pak Choi during modified atmosphere storage. *Food Control.* 100, 17–23 (2019).
106. Mohd Zain, N. & Bakar, L. Eugenol, an essential oil of clove, causes disruption to the cell wall of *Candida albicans*. *Front. Life Sci.* 8(3), 231–240 (2015).
107. Cui, H., Ma, C. & Lin, L. Synergetic antibacterial efficacy of cold nitrogen plasma and clove oil against *Escherichia coli* O157:H7 biofilms on lettuce. *Food Control.* 66, 8–16 (2016).
108. Sharma, M., Mann, B., Pothuraju, R., Sharma, R. & Kumar, R. Physico-chemical characterization of ultrasound assisted clove oil-loaded nanoemulsion: as enhanced antimicrobial potential. *Biotechnol. Rep.* 34, e00720 (2022).
109. Sharma, S., Singh, S., Bond, J., Singh, A., & Rustagi, A. Evaluation of antibacterial properties of essential oils from clove and eucalyptus. *Evaluation*, 7(5), (2014).
110. Sharma, S., Barkauskaite, S., Duffy, B., Jaiswal, A. K. & Jaiswal, S. Characterization and antimicrobial activity of biodegradable active packaging enriched with clove and thyme essential oil for food packaging application. *Foods* 9 (8), 1117 (2020).
111. Milagres de Almeida, J. et al. Perez Alonso VP, da Motta Santos Júnior E, Siqueira Franco Picone C., Antimicrobial action of Oregano, Thyme, Clove, Cinnamon and Black pepper essential oils free and encapsulated against foodborne pathogens. *Food Control.* ;144:109356. (2023).
112. Jantan, I., Karim Moharam, B. A., Santhanam, J. & Jamal, J. A. Correlation between chemical composition and antifungal activity of the essential oils of eight cinnamomum. *Species Pharm. Biol.* 46 (6), 406–412 (2008).
113. Pandey, D. K. et al. Current Knowledge of Cinnamomum Species: A Review on the Bioactive Components, Pharmacological Properties, Analytical and Biotechnological Studies. In: Singh J, Meshram V, Gupta M, editors. *Bioactive Natural products in Drug Discovery* [Internet]. Singapore: Springer; [cited 2025 Feb 8]. pp. 127–64. (2020). Available from: https://doi.org/10.1007/978-981-15-1394-7_3
114. Sriramavaratharajan, V. & Murugan, R. Chemical profile of leaf essential oil of cinnamomum Walaiwarens and comparison of its antioxidant and hypoglycemic activities with the major constituent benzyl benzoate. *Nat. Prod. Commun.* 13 (6), 1934578X1801300633 (2018).
115. Bonilla, J. & Sobral, P. J. A. Investigation of the physicochemical, antimicrobial and antioxidant properties of gelatin-chitosan edible film mixed with plant ethanolic extracts. *Food Biosci.* 16, 17–25 (2016).
116. Zhang, Y., Liu, X., Wang, Y., Jiang, P. & Quek, S. Antibacterial activity and mechanism of cinnamon essential oil against *Escherichia coli* and *Staphylococcus aureus*. *Food Control.* 59, 282–289 (2016).
117. Abdeldaiem, M. H. M., Ali, H. G. M. & Ramadan, M. F. Impact of different essential oils on the characteristics of refrigerated carp (*Cyprinus carpio*) fish fingers. *J. Food Meas. Charact.* 11 (3), 1412–1420 (2017).
118. Muneke, P. E. S. et al. The role of essential oils against pathogenic *Escherichia coli* in food products. *Microorganisms* 8 (6), 924 (2020).
119. Sheng, L., Rasco, B. & Zhu, M. J. (ed Dudley, E. G.) Cinnamon oil inhibits Shiga toxin type 2 phage induction and Shiga toxin type 2 production in *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 82 22 6531–6540 (2016).
120. Yuan, W., Teo, C. H. M. & Yuk, H. G. Combined antibacterial activities of essential oil compounds against *Escherichia coli* O157:H7 and their application potential on fresh-cut lettuce. *Food Control.* 96, 112–118 (2019).
121. Raeisi, M., Tabaraei, A., Hashemi, M. & Behnampour, N. Effect of sodium alginate coating incorporated with nisin, *Cinnamomum zeylanicum*, and Rosemary essential oils on microbial quality of chicken meat and fate of *Listeria monocytogenes* during refrigeration. *Int. J. Food Microbiol.* 238, 139–145 (2016).
122. Wen, P. et al. Fabrication of electrospun polylactic acid nanofilm incorporating cinnamon essential oil/ β -cyclodextrin inclusion complex for antimicrobial packaging. *Food Chem.* 196, 996–1004 (2016).
123. Adesiji, Y. et al. *Niger J. Pure Appl. Sci.* ;28:2610–2616. (2015).
124. Arakeeb, S. M., Hassanien, F., Shaltout, F. & Homouda, S. Natural preservatives in Raw chicken meat. *Benha Vet. Med. J.* 37 (1), 41–45 (2019).

125. Nychas, G. J. E. Natural antimicrobials from plants. In: Gould GW, editor. *New Methods of Food Preservation* [Internet]. Boston, MA: Springer US; [cited 2025 Feb 8]. pp. 58–89. (1995). Available from: https://doi.org/10.1007/978-1-4615-2105-1_4
126. Casalino, G. et al. Antimicrobial efficacy of cinnamon essential oil against avian pathogenic *Escherichia coli* from poultry. *Animals* **13** (16), 2639 (2023).
127. Efenberger-Szmechtyk, M., Nowak, A. & Czyzowska, A. Plant extracts rich in polyphenols: antibacterial agents and natural preservatives for meat and meat products. *Crit. Rev. Food Sci. Nutr.* **61** (1), 149–178 (2021).
128. Santiesteban-López, A., Palou, E. & López-Malo, A. Susceptibility of food-borne bacteria to binary combinations of antimicrobials at selected a(w) and pH. *J. Appl. Microbiol.* **102** (2), 486–497 (2007).
129. Abdelli, N., Solà-Oriol, D. & Pérez, J. F. Phytogenic feed additives in poultry: achievements, prospective and challenges. *Animals* **11** (12), 3471 (2021).
130. Ali, A. et al. Cinnamon: A natural feed additive for poultry health and Production—A review. *Animals* **11** (7), 2026 (2021).
131. Ali, S. M. et al. Antimicrobial activities of Eugenol and cinnamaldehyde against the human gastric pathogen *Helicobacter pylori*. *Ann. Clin. Microbiol. Antimicrob.* **4** (1), 20 (2005).

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Declarations

Competing interests

The authors declare no competing interests.

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