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Antibacterial activity of plant-derived product against avian pathogenic Escherichia coli isolated from laying hens with colibacillosis: Influence of resistance profiles, synergy assessment, and validation in complex biological matrices

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Abstract

The global emergence of antimicrobial-resistant bacteria has intensified the search for sustainable alternatives to conventional antimicrobial drugs in animal production. Among these alternatives, plant-derived compounds, including essential oils and plant extracts, have gained increasing attention due to their antimicrobial properties. However, their efficacy against avian pathogenic *Escherichia coli* (APEC) from laying hens remains poorly characterized. The objective of this study was to evaluate the antibacterial activity of a commercial plant-derived product against multiple APEC strains isolated from laying hens diagnosed with colibacillosis and exhibiting diverse antimicrobial resistance profiles. Additionally, the antibacterial activity of individual essential oils and their combinations with a plant extract was evaluated to identify potential synergistic effects and determine their contribution to the overall efficacy of the product. Sixteen clinical APEC strains recovered from laying hens with colibacillosis were whole-genome sequenced, and their phenotypic and genotypic antimicrobial resistance (AMR) profiles were characterized. The minimum inhibitory concentrations (MICs) of the commercial plant-derived product were determined for all APEC strains in a simple matrix consisting of pure bacterial cultures. Six APEC representing distinct AMR profiles were subsequently selected to assess product efficacy in a complex biological matrix composed of cecal content from laying hens inoculated with an APEC strain. The antibacterial activity of individual components was characterized by MIC determination, and the interactions between combined components were evaluated using the fractional inhibitory concentration index (FICI) to determine their potential synergistic contribution to the overall efficacy of the formulation. The commercial product demonstrated consistent antibacterial activity against all tested APEC strains, regardless of their AMR profiles. Compared with pure bacterial cultures, its antibacterial activity was reduced by one dilution in the complex cecal matrix. Among individual components, cinnamon and oregano essential oils exhibited the strongest antibacterial activity, while clove essential oil showed lower activity and pomegranate extract displayed APEC-dependent effects. No synergistic or antagonistic interactions ($0.8 < FICI < 4$) were observed among the tested combinations. These results highlight the potential of a plant-derived formulation as an alternative to conventional antimicrobials for managing antimicrobial-resistant APEC infections. Future studies using an *in vivo* colibacillosis model in laying hens are needed to validate these *in vitro* findings and determine their potential role in antimicrobial stewardship strategies.

Introduction

Antimicrobial resistance (AMR) represents one of the most critical global challenges for human, animal, and environmental health [1]. The emergence and dissemination of antimicrobial-resistant bacteria (ARB) threaten the effectiveness of existing therapeutic options and compromise the ability to control bacterial infections in both human and veterinary medicine [2]. Food-producing animals have been pointed out as important contributors to the selection and dissemination of ARB and may serve as a major route for the introduction of these bacteria into the food chain [3]. Consequently, reducing the reliance on conventional antimicrobials in livestock and poultry production has become a priority, requiring the development of effective and sustainable alternative strategies to prevent and control

bacterial diseases [4]. On the other hand, despite considerable efforts in poultry production to strengthen preventive strategies, such as biosecurity, vaccination, nutritional interventions and improved husbandry practices, bacterial infections remain a major concern, leading to substantial economic losses, reduced productivity, and persistent challenges in animal health management [5]

Among bacterial diseases affecting poultry production, avian colibacillosis caused by avian pathogenic *Escherichia coli* (APEC) remains one of the most important infectious diseases worldwide [6]. APEC associated with colibacillosis in poultry predominantly belong to serogroups O78, O2, and O1, although other serogroups such as O25 and O111 may also be involved depending on the geographic region and production system [7–9]. Although APEC infections can affect various poultry production systems, laying hens represent an important production sector in which colibacillosis can have substantial health and economic consequences. In particular, APEC infections can lead to significant reductions in egg production, with losses of up to 70% reported during severe outbreaks [10]. The economic impact of these infections, combined with their potential to affect hens throughout the production cycle, makes colibacillosis an important concern in laying operations. Colibacillosis can occur throughout the rearing and laying periods, with incidence increasing during peak egg production and toward the end of the production cycle [11]. In laying hens, APEC can cause localized or systemic colibacillosis, with clinical manifestations ranging from nonspecific signs to sudden mortality [12]. Affected birds may exhibit depression, reduced feed intake, respiratory distress, decreased egg production, and a sudden increase of mortality [13]. Gross lesions commonly include airsacculitis, pericarditis, perihepatitis, salpingitis, oophoritis, and peritonitis with yolk material deposited in the peritoneal cavity [9, 13, 14]. The control of APEC-associated infections relies largely on antimicrobial treatments; however, the increasing prevalence of antimicrobial-resistant APEC has compromised therapeutic efficacy and raised concerns regarding the sustainability of current disease management practices [15]. In laying hens, antimicrobial treatment also presents an important production constraint, as many antimicrobials require an egg withdrawal period to prevent residues from entering the food chain. Consequently, eggs produced during this period cannot be marketed, resulting in additional production and economic losses [16]. These limitations further highlight the need for effective alternative or complementary strategies for the control of APEC-associated infections in laying hens. Moreover, antimicrobial treatment in laying hens can result in drug residues in eggs, requiring withdrawal periods that may lead to economic losses from unsaleable eggs [17]. These limitations underscore the need to develop effective non-antimicrobial strategies for APEC control that can reduce reliance on conventional antimicrobials and support antimicrobial stewardship in laying-hen production.

Plant-derived compounds, including essential oils (EOs) and plants extracts, have emerged as promising alternatives to conventional antimicrobials for the prevention and control of bacterial infections in poultry [18, 19]. These natural compounds exhibit diverse antibacterial properties through multiple mechanisms of action, including disruption of bacterial cell membranes, alteration of cellular permeability, inhibition of the efflux pump, and interference with essential metabolic processes (e.g., interact with nucleic acids) [18, 20]. Several EOs, particularly those derived from oregano (*Origanum vulgare*) and cinnamon (*Cinnamomum* spp.), have demonstrated inhibitory activity against a broad range

of bacterial pathogens, including *E. coli, Staphylococcus aureus*, *Pseudomonas aeruginosa* [20], and have therefore attracted increasing interest as potential tools to support antimicrobial stewardship in poultry production [21, 22]. Other plant-derived compounds, such as clove (*Syzygium aromaticum*) essential oil and pomegranate (*Punica granatum*) extracts, have also shown antimicrobial potential, either as individual components or in combination with other bioactive molecules [23–25]. The antimicrobial activity of oregano, cinnamon, and clove essential oils, as well as pomegranate extracts, is mainly attributed to their key bioactive compounds: carvacrol/thymol, cinnamaldehyde, eugenol, and punicalagin, respectively [26, 27]. However, despite increasing evidence supporting the potential of plant-derived antimicrobials as alternatives to conventional antimicrobials in poultry production, most studies have been conducted in broiler chickens or using laboratory strains [28], with limited investigation in laying hens and field-relevant pathogens affecting this production sector. Furthermore, the ability of these compounds to maintain their antibacterial activity against strains with different AMR profiles, as well as under complex and biologically relevant conditions, remains poorly documented. Numerous studies, conducted predominantly under *in vitro* conditions, have demonstrated the antibacterial activity of EOs against a broad range of resistant pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum β-lactamase (ESBL)-producing *E. coli*, multidrug-resistant *Pseudomonas aeruginosa*, and other clinically and veterinary relevant AMR bacteria [29–32]. However, to the best of our knowledge, no study has systematically investigated whether phenotypic AMR profiles or specific antimicrobial resistance genes (ARGs) affect bacterial susceptibility to plant-derived products. Therefore, this study aimed to (i) evaluate the antibacterial activity of a commercially available plant-derived formulation against APEC strains isolated from laying hens with colibacillosis; (ii) determine whether phenotypic and genotypic AMR profiles influence APEC susceptibility to the formulation; and (iii) investigate the individual and combined antibacterial activities of its major essential oil constituents and plant extract to identify potential synergistic interactions and elucidate their contribution to the formulation's overall antibacterial activity.

Materials and methods

1. Bacterial strains

A total of 16 APEC strains were included in this study (Table 1). These APEC were recovered from laying hens diagnosed with colibacillosis and were previously characterized as APEC based on the presence of five major virulence-associated genes commonly identified in highly pathogenic APEC strains, as described by Johnson T et al. [33]. All APEC were obtained from the World Organisation for Animal Health (WOAH) Reference Laboratory for *E. coli* (EcL) at the Faculty of veterinary medicine (FVM) of the Université de Montréal. The 16 APEC strains were selected to maximize the diversity of the bacterial collection. In fact, these strains originated from different birds, buildings, and sampling years, thereby minimizing the likelihood of including epidemiologically related isolates and allowing the antibacterial activity of the plant-derived product to be evaluated across a diverse range of APEC strains.

2. Phenotypic and genotypic antimicrobial resistance profiling

The phenotypic antimicrobial susceptibility profiles of the 16 APEC strains were determined using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar (Fisher Scientific, Ottawa, ON, Canada), following established guidelines [34]. Briefly, bacterial inocula were prepared from fresh overnight cultures suspended in sterile saline (0,85% NaCl) and adjusted to a turbidity equivalent to a 0.5 McFarland standard. The standardized suspensions were uniformly inoculated onto Mueller–Hinton agar plates, after which antimicrobial-impregnated disks were applied to the agar surface. Plates were incubated aerobically at 37 °C for 18–24 h.

The antimicrobial agents tested were selected based on their relevance and frequent use in the Canadian poultry production context and represented the major antimicrobial classes used by this industry. The panel included ceftiofur (30 µg), ampicillin (10 µg), gentamicin (10 µg), tobramycin (10 µg), streptomycin (10 µg), kanamycin (25 µg), sulfamethoxazole–trimethoprim (23.75/1.25 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), and oxytetracycline (30 µg).

Following incubation, inhibition zone diameters were measured in millimeters and interpreted as susceptible, intermediate, or resistant according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [35], and Clinical and Laboratory Standards Institute (CLSI) M100 35^{th} Edition, 2025 guidelines [36]. APEC were classified as multidrug-resistant (MDR) when they exhibited non-susceptibility to at least one antimicrobial agent in three or more antimicrobial classes, in accordance with internationally accepted definitions [37]. It is important to note that the laboratory reference strain *E. coli* ATCC 25922 was included as a quality-control strain for the disk diffusion antimicrobial susceptibility testing. Results obtained for the control strain were compared with the established CLSI quality-control ranges for each antimicrobial tested.

The genotypic antimicrobial resistance profiles of all 16 APEC strains were characterized based on the identification of ARGs from whole-genome sequencing (WGS) data, as previously described **[38]**. Briefly, genomic DNA was extracted from bacterial cultures incubated overnight using the QIA Symphony automated extraction platform and the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. Sequencing libraries were prepared using the Illumina DNA kit (formerly the Nextera Flex Kit) (Illumina Inc., San Diego, CA, USA) and sequenced on the Illumina (San Diego, CA) iSeq100 platform with 2 × 150 reads (Illumina Inc., San Diego, CA, USA) [39].

Genotypic AMR profiles were characterized by identifying ARGs from WGS data using the ResFinder tool available on the bioinformatics platform of the Center for Genomic Epidemiology at the Technical University of Denmark (https://genepi.food.dtu.dk/resfinder), which was used to identify acquired AMR genes from the assembled genome sequences.

3. Commercial plant-derived product

The plant-derived product (Phytosynthese, France) used in the present study consisted of a blend of EOs rich in trans-cinnamaldehyde, eugenol, carvacrol, and thymol, combined with plant extracts including pomegranate, garlic, and green propolis extract (Table 2). The quantification of these bioactive

compounds was conducted by the Phytosynthese Laboratory using either high-performance liquid chromatography (HPLC) or gas chromatography (GC), as appropriate for each component, as previously described [40,41].

4. Antibacterial characterization of the commercial plant-derived product

a- Evaluation of the antibacterial activity against the APEC strains in pure culture

The antibacterial activity of the commercial plant-derived product was evaluated using the macrodilution (tube dilution) method, as described by Wiegand et al. [42]. The 16 APEC strains were cultured on blood TSA (Trypticase Soy Agar supplemented with 5% sheep blood) (OXOID, Ottawa, Canada) and incubated at 37 °C for 18 to 24 h.

Bacterial suspensions were adjusted to a turbidity of 0.5 McFarland standard (approximately $1–2 \times 10^8$ CFU/mL) [43]. A subsequent 1:10 dilution was performed in Mueller–Hinton broth (MHB) (Fisher Scientific, Le Pont de Claix, France) to obtain a final inoculum concentration of approximately 5×10^7 CFU/mL. An aliquot of 500 µL of the diluted bacterial suspension was then added to each tube for the determination of the minimum inhibitory concentration (MIC).

The commercial plant-derived product was first diluted in MHB, then subjected to serial two-fold dilutions in the same medium to obtain final concentrations ranging from 1.5% to 0.04% (v/v) of commercial plant-derived product. For each concentration tested, 4.5 mL of the corresponding dilution was transferred to a sterile tube, and then 500 µL of the previously prepared bacterial suspension was added, resulting in a final volume of 5 mL per tube.

For each assay, a positive control consisting of the bacterial suspension without any addition of EOs was included. A negative control was also included, containing only MHB, with no bacterial suspension or other product.

The tubes were incubated for 24 h at 37 °C under constant agitation (200 rpm) using a rotary shaker. The MIC was defined as the lowest concentration of commercial plant-derived product that inhibited visible bacterial growth, as indicated by the absence of turbidity following incubation, all assays were performed in triplicate technical and biological.

b- Evaluation of the antibacterial activity of the commercial plant-derived product in laying hen cecal content as a complex biological matrix

To evaluate the antibacterial activity of the plant-derived product in the presence of a complex and highly diverse microbial community, cecal contents were collected from 70-week-old spent laying hens at a commercial slaughterhouse in Quebec, Canada. Upon arrival at the laboratory, all samples were pooled, weighed and mixed with an equal volume of freezing medium (1:1, w/v). The resulting suspension was thoroughly homogenized and stored at -80°C until further use.

Six APEC strains representing different AMR profiles were selected for this study. *E. coli* ATCC 25922 was included as a quality-control strain. To enable selective recovery and enumeration within the complex cecal microbiota, all six APEC were rendered resistant to nalidixic acid through passages on MacConkey agar supplemented with 25µg/mL concentrations of nalidixic acid. This selective marker allowed the differentiation of the inoculated APEC strains from the indigenous cecal bacterial population during microbiological analyses.

Separately, the six nalidixic acid-resistant (NAL-R) APEC strains were prepared using the same protocol described above, except that 70 µL of cecal content was added to each tube after a preliminary 1:10 dilution of the cecal content in MHB, as previously described [44]. All tubes were incubated for 24 h at 37 °C under constant agitation (200 rpm) using a rotary shaker. Following incubation, an aliquot (10 µL) from each tube was plated on MacConkey agar supplemented with nalidixic acid (25 mg/L) to selectively recover the nalidixic acid-resistant APEC strains. Plates were incubated for an additional 24 h at 37 °C, and bacterial growth was assessed. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of a commercial plant-derived product that resulted in no detectable growth of the NAL-R APEC strains following subculture onto agar plates and incubation under appropriate conditions. MBC determination was therefore based on the presence or absence of visible bacterial growth on the agar surface, with the absence of growth indicating a bactericidal effect rather than merely bacterial growth inhibition. To ensure that the agar plates were free of contamination, two controls were prepared: a negative control, containing no commercial plant-derived product, no APEC, and no cecal contents; and a control containing the NAL-R APEC strain but no commercial plant-derived product and no cecal contents. All experiments were conducted in triplicate technical and biological.

5. Assessment of the antibacterial activity of certain essential oils and pomegranate extract, key components of a plant-derived product, against antimicrobial-resistant APEC strains

a- Individual antibacterial activity of essential oils and pomegranate extract against antimicrobial-resistant APEC

To investigate potential synergistic interactions between selected EOs and pomegranate extract, the antimicrobial activity of each component was evaluated individually against the six APEC strains previously selected in the preceding section. The EOs tested included cinnamon, oregano, and clove, essential oils, each formulated at a concentration of 9% using appropriate solubilizing agents to ensure homogeneous dispersion in the culture medium. The pomegranate extract was supplied as a dry powder and prepared accordingly prior to antimicrobial testing.

Serial dilutions of each compound were prepared in Mueller-Hinton broth. Essential oils were tested over a concentration range of 0.04% to 1.5%, corresponding to the dilution series previously employed for the pure culture assay. As a reminder, the essential oils used were 9% concentrated and contained solubilizing agents. Pomegranate extract, supplied in powder form, was reconstituted prior to testing and evaluated at concentrations of 0.39%, 0.50%, 0.78%, 1.00%, 1.50%, 2.00%, and 2.50%. For each tested concentration tested, 4.5 mL of the prepared solution was dispensed into sterile test tubes.

Separately, the six APEC strains were prepared using the same protocol described above. All tubes were incubated for 24 hours at 37 °C under constant agitation (200 rpm) using a rotary shaker. The MIC was defined as the minimum concentration of essential oils that prevented any visible bacterial growth (no observable turbidity after incubation). For EOs that produced substantial intrinsic turbidity, which prevented reliable visual discrimination between bacterial growth and oil-associated turbidity, the MBC was defined as the lowest concentration of EOs that prevented any visible bacterial growth (the concentration at which no growth was observed on agar plates), as described in the previous section. All experiments were conducted using three biological replicates, each with three technical replicates.

b- Evaluation of synergistic interactions among essential oils and pomegranate extract against antimicrobial-resistant APEC strains

The potential synergistic interactions between the tested EOs (cinnamon, oregano, or clove) and pomegranate extract were evaluated using a checkerboard method, which involves determining the fractional inhibitory concentration index (FICI), as described previously by Lv et al. and Zekri et al. [38,45]. The test was conducted in two successive steps. First, the concentration of the pomegranate extract was maintained at a fixed subinhibitory concentration corresponding to a dilution below the previously determined MIC, while serial dilutions of the EOs (cinnamon, oregano, clove, or garlic) were prepared. The concentration of each EO was gradually reduced from the MIC until visible bacterial growth was observed. In the second step, the procedure was reversed. The concentration of the EO was maintained at a fixed subinhibitory concentration corresponding to a dilution below the MIC, while the concentration of the pomegranate extract was serially diluted until bacterial growth was detected.

For each concentration tested, 4.5 mL of the prepared solution was dispensed into sterile test tubes. Bacterial suspensions from each of the seven APEC previously selected in the preceding section were standardized to a turbidity equivalent to a 0.5 McFarland standard and subsequently diluted 1:10 in MHB. An aliquot of 0.5 mL of the diluted inoculum was then added to each tube. All tubes were incubated for 24 h at 37 °C under constant agitation (200 rpm) using a rotary shaker. Following incubation, an aliquot (10 µL) from each tube was plated on TSA and incubated for an additional 24 h at 37 °C. All experiments were performed in triplicate to ensure the reproducibility and reliability of the results.

The interaction between EOs and pomegranate extract was evaluated using the FICI, a widely used parameter for assessing the synergistic, additive, indifferent, or antagonistic effects of antimicrobial combinations [46]. The FICI was calculated as follows:

$FICI = FIC_A + FIC_B$

Where FIC_A and FIC_B represent the fractional inhibitory concentrations of compounds A and B, respectively, calculated as:

FIC_A = MIC of compound A in combination / MIC of compound A alone;

FIC_B = MIC of compound B in combination / MIC of compound B alone.

Based on the criteria proposed by Ngolo et al. [47], interactions were interpreted as follows: FICI ≤ 0.5 indicated a synergistic interaction, partial synergy (0.5 < FICI ≤ 0.75), additive (0.75 < FICI ≤ 1.0), indifferent (1.0 < FICI ≤ 4.0), or antagonistic between the tested compounds (FICI > 4.0).

6. Genomic characterization of APEC strains

Whole-genome sequence data were further analyzed to characterize the genetic background of the six APEC strains, representing distinct AMR profiles, that were selected for the combination assays. Multilocus sequence typing (MLST) and phylogroup assignment were determined for these strains (Table 3). The AMR-associated genes identified previously, as described in the section "Phenotypic and genotypic antimicrobial resistance profiling," were also considered in the genomic characterization. These genomic features were descriptively compared with the MIC values of the complete plant-derived formulation and the FICI-based interaction profiles obtained for these selected strains. Given the limited number of strains (n=6), these analyses were considered exploratory and were not intended to establish statistically validated genotype–phenotype associations.

Results

1. Antimicrobial resistance profiles of the APEC strains (n=16)

a- Phenotypic antimicrobial susceptibility profiles of APEC

The antimicrobial susceptibility profiles of the 16 APEC were determined using the disk diffusion method. Overall, resistance varied across antimicrobial classes, with the highest prevalence observed among aminoglycosides and tetracyclines (Figure 1). Indeed, resistance to gentamicin was the most prevalent, with 10/16 isolates (62.5%) classified as resistant, followed by streptomycin (8/16, 50.0%), oxytetracycline (7/16, 43.8%), and tobramycin (6/16, 37.5%). Resistance to kanamycin was observed in 5/16 APEC (31.3%). Resistance to trimethoprim-sulfamethoxazole was detected in 2/16 APEC strains (12.5%). Among the β-lactams tested, 8/16 APEC (50.0%) were resistant to ampicillin, whereas no resistance to ceftiofur was observed; one strain (6.3%) showed intermediate susceptibility. All isolates were susceptible to ciprofloxacin, while resistance to nalidixic acid was detected in 1/16 APEC (6.3%). According to the criteria defined in this study, 6/16 APEC (37,5%) were classified as MDR (Table 4). These MDR strains exhibited resistance to antimicrobials belonging to multiple classes, including β-lactams, aminoglycosides, trimethoprim-sulfamethoxazole, and tetracyclines.

B- Distribution of antimicrobial resistance genes among the APEC strains

Whole-genome sequencing identified several ARGs genes among the 16 APEC strains (Table 4). The most frequently detected determinants included bla_{TEM-1B}, *aac(3)-IId*, *aac(3)-VIa*, *sul1*, *tet(A)*, and *tet(B)*. Among β-lactam resistance genes, bla_{TEM-1B} was detected in 6/16 isolates (37.5%), while bla_{CMY-2} was

identified in 3/16 isolates (18.8%). Several genes encoding aminoglycoside-modifying enzymes were detected, including *aac(3)-IId*, *aac(3)-VIa*, *aadA1*, *aph(6)-Id*, *aph(3")-Ib*, and *aph(3')-Ia*. Folate pathway inhibitors resistance determinants included *sul1*, *sul2*, and *dfrA15*. Tetracycline resistance was associated with *tet(A)* and *tet(B)*, with *tet(A)* detected more frequently among oxytetracycline-resistant isolates. On the other hand, several amino acid substitutions were identified in quinolone target genes, notably *gyrA* (D678E and A828S), *gyrB* (S492N and A618T), and *parC* (M241I, D475E, and T718A).

2. Antibacterial activity of the plant-derived product against APEC strains in pure culture and complex matrix

The antibacterial activity of the plant-derived product was initially evaluated by determining the MICs against all 16 APEC strains in pure culture. The activity was subsequently assessed against six APEC strains selected according to their AMR profiles in a complex cecal matrix containing pooled cecal contents inoculated with each respective APEC strain (Table 5).

Under pure culture conditions, an MIC of 7800 µg/mL was observed for all tested strains, including the reference strain *E. coli* ATCC 25922, regardless of their AMR profiles. In contrast, in the complex cecal matrix, a one-dilution decrease in antibacterial activity was observed, with the MIC increasing from 7800 to 15625 µg/mL for all six selected APEC strains as well as the ATCC 25922 control strain and this MIC value remained consistent across the tested APEC, irrespective of their AMR profiles (Table 5).

3. Antibacterial activity of essential oil–pomegranate extract combinations against the selected APEC strains (n=6)

The antimicrobial activity of cinnamon, oregano, and clove essential oils, as well as pomegranate extract, was assessed by determining their MICs individually and in combination against six antimicrobial-resistant APEC and *E. coli* ATCC 25922 field (Table 6). When tested individually, each essential oil exhibited consistent MIC values, the values were 3900 µg/mL for cinnamon and oregano, and 15625 µg/mL for clove, across all tested strains, including *E. coli* ATCC 25922 and the six APEC strains (Table 6). However, the MICs differed among the EOs evaluated. Cinnamon and oregano EOs demonstrated the greatest antibacterial activity, with MIC values of 3900 µg/mL, whereas clove essential oil exhibited a MIC of 15625µg/mL. Similarly, the MICs of pomegranate extract were consistent across all tested strains, ranging from 10000 to 20000 µg/mL (Table 6).

The combination of cinnamon essential oil and pomegranate extract reduced the MIC of cinnamon for three of the seven strains tested (ECL24324, ECL20552, and ECL23871), yielding an FIC index of 0.5. A pronounced effect was observed for strain ECL20719, for which the MIC decreased by 4 dilutions and the FIC of cinnamon decreased to 0.1. The FICI values for the combination of cinnamon essential oil and pomegranate extract ranged from 0.8 to 1.8 across the tested strains. Based on the predefined criteria [46], these values corresponded to either additive ($0.75 < FICI \leq 1$) or non-interactive ($1 < FICI \leq 4$) effects. No synergistic or antagonistic interactions were observed (Table 3).

The combination of oregano and pomegranate, unlike cinnamon, did not result in any decrease in the MIC of oregano essential oil. FIC values for oregano remained constant at 1.0 for all strains. FICI values for the combination of oregano essential oil and pomegranate extract ranged from 1 to 3 across the tested strains (Table 6).

The combination of clove and pomegranate resulted in a 50% reduction (one dilution) in the MIC of clove for all tested APEC, as indicated by the constant FIC values of 0.5. The FICI values for the combination of clove essential oil and pomegranate extract ranged from 1 to 1.5 across the tested strains. The lowest values (FICI = 1.0) were observed for strains ECL24324, ECL21752, and ECL24026, indicating an additive interaction between clove essential oil and pomegranate extract. In contrast, the remaining strains exhibited FICI values of 1.5, corresponding to a non-interactive effect. Overall, no synergistic (FICI ≤ 0.5) or antagonistic (FICI > 4) interactions were observed between the two components (Table 6). Among the three combinations evaluated, the cinnamon-pomegranate combination exhibited the lowest FICI values, particularly for strain ECL20719 (FICI = 0.8), indicating an additive interaction. The clove-pomegranate combinations yielded intermediate FICI values ranging from 1.0 to 1.5, corresponding to additive to non-interactive effects (Table 6). In contrast, the oregano-pomegranate combination exhibited the highest FICI values, reaching 3.0, and was therefore classified as non-interactive. Overall, none of the combinations demonstrated a synergistic effect under the experimental conditions tested in the present study (Table 6).

The analysis of the molecular characteristics of the selected APEC strains revealed a predominance of additive interactions among strains belonging to phylogroup C. In particular, ECL 24324, ECL 24026, and ECL 21752, all of which belonged to ST23 and serotype O78, exhibited additive interactions with the clove–pomegranate extract combination (Supplementary Table 1). In addition, the ECL 24324 strain also showed an additive interaction with the cinnamon–pomegranate extract combination, whereas the ECL 20719 strain, another O78 strain belonging to ST9862, exhibited an additive interaction with the same combination (Supplementary Table 1).

Discussion

The objective of this study was to characterize the antibacterial activity of a plant-based product against clinical strains of APEC isolated from laying hens in which colibacillosis had been diagnosed and for which we determined various phenotypic and genotypic profiles of AMR. The antibacterial activity of the plant-derived product was initially evaluated under standard culture conditions and subsequently in a complex cecal matrix to better assess its activity in a biologically relevant environment. In addition, the antibacterial activities of selected individual EOs and pomegranate extract were evaluated, and their interactions were characterized using the FICI method. To the best of our knowledge, this study is the first to evaluate the antibacterial activity of a plant-derived product against clinical APEC strains with diverse AMR profiles and recovered from laying hens, under both pure culture conditions and in a complex cecal matrix. Furthermore, this study provides the first systematic characterization of the

interactions between selected EOs and a commercial pomegranate extract against APEC strains, providing insights into their potential for targeting AMR bacteria in complex biological matrices.

The antimicrobial susceptibility profiles of the 16 clinical APEC strains revealed substantial variation in resistance across antimicrobial classes. However, the AMR profile observed in the present study should be interpreted in light of the relatively small number of strain and their specific epidemiological origin (Québec, Canada). In addition, unlike many previous studies that have primarily investigated APEC isolates from broiler chickens, the isolates examined in the present study were recovered from clinical cases of colibacillosis in laying hens. This distinction is important, as antimicrobial use practices, production systems, and epidemiological characteristics may differ substantially between broiler chicken and egg production in Canada, potentially influencing the distribution of AMR among APEC populations [48].

Overall, resistance was most prevalent to aminoglycosides and tetracyclines among the APEC strains included in this study. Gentamicin exhibited the highest resistance rate (62.5%), followed by streptomycin (50%), while resistance to oxytetracycline was observed in 43.8% of the isolates. These findings are consistent with previous studies reporting high frequencies of resistance to aminoglycosides and tetracyclines among APEC isolates recovered from poultry. For example, Zhao et al. reported high levels of antimicrobial resistance among 95 APEC isolates recovered from clinical cases of colibacillosis in broiler chickens in North Georgia, USA, with resistance rates of 69% to gentamicin, 86% to streptomycin, and 87% to tetracycline [49]. In contrast, resistance to trimethoprim-sulfamethoxazole was detected in only 12.5% of APEC, while all strains were susceptible to ciprofloxacin and ceftiofur. These findings are consistent with previous studies conducted on *E. coli* isolates recovered from laying hens [50]. For instance, Gambi et al. (2022), who characterized 71 *E. coli* strains isolated during outbreaks of colibacillosis in laying hens in Italy, reported no resistance to ceftiofur and similarly low levels of resistance to trimethoprim-sulfamethoxazole [51]. Collectively, these findings suggest that resistance to critically important antimicrobial classes remain relatively uncommon among APEC isolates associated with clinical colibacillosis in laying hens in Québec. Similarly, recent reviews of AMR in APEC suggest that resistance rates among isolates from laying hens tend to be lower than those reported in other poultry production systems, particularly broiler chicken production, although differences in study design, antimicrobial susceptibility testing methods, geographic origin, and antimicrobial use practices should be considered when making such comparisons [11, 52, 53].

At the genomic level, WGS identified several ARGs, and their distribution was consistent with the phenotypic resistance profiles, particularly for β-lactams, aminoglycosides, and tetracyclines, in agreement with previous reports of these determinants in APEC isolates [54, 55].

A major finding of the current study was the consistent antibacterial activity of the plant-derived product against all tested strains, including the reference strain *E. coli* ATCC 25922 and the 16 clinical APEC strains. Under pure culture conditions, a MIC of 7800 µg/mL was observed across all APEC, regardless of their phenotypic or genotypic AMR profiles. This result suggests that the antibacterial activity of the

product was not substantially influenced by the AMR determinants identified in the APEC collection, indicating that the AMR mechanisms characterized in this study did not confer cross-resistance to the plant-derived formulation. It is noteworthy that APEC strains are known to exhibit significant genetic variability as well as heterogeneous virulence and resistance profiles that can influence their response to antimicrobial agents [56]. Despite this diversity, the lack of variation in MICs observed in the present study suggests that the plant-derived product acts similarly on all tested strains, indicating a stable intrinsic activity [57]. In fact, previous studies have similarly reported that the antibacterial activity of EOs is often mediated through multiple cellular targets, thereby reducing the likelihood that a single AMR mechanism would compromise their efficacy [58]. In addition, a comprehensive review by Iskandar et al. highlighted the broad-spectrum antibacterial activity of several EOs, particularly those rich in carvacrol, thymol, cinnamaldehyde, and eugenol, against clinically relevant MDR pathogens (e.g., *E. coli*, *P. aeruginosa*, *K. pneumoniae*) [32]. In the current study, the activity of the commercial plant-derived product may be less affected by the resistance mechanisms identified in the present APEC collection than are antimicrobials. Nevertheless, additional studies involving larger collections of APEC stains exhibiting a broader diversity of AMR determinants are required to confirm this observation.

To better assess the potential efficacy of the plant-derived product under complex microbial conditions characterized by a high and diverse microbial load, its antibacterial activity was then evaluated in a complex cecal matrix. To the best of our knowledge, this study is the first to investigate the antibacterial activity of a phytogenic product against clinical APEC strains covered from laying hens using both standard *in vitro* culture conditions and a complex cecal-content matrix. Compared with the MIC observed in pure culture (7800 µg/mL), a one-fold reduction in antibacterial activity was observed in the presence of cecal contents, resulting in a MIC of 15625 µg/mL for all the six selected APEC strains and the reference strain *E. coli* ATCC 25922. Although modest, this decrease suggests that components of the cecal matrix may influence the antibacterial activity of phytogenic compounds. In particular, interactions with proteins, lipids, and other organic components of the matrix may reduce the free concentration or bioavailability of hydrophobic phytogenic compounds, such as essential-oil constituents, thereby attenuating their antibacterial activity [59]. However, the specific interactions underlying this reduction remain to be determined. On the other hand, these findings open promising perspectives for future *in vivo* investigations to further assess the potential of this plant-derived product as an alternative or complementary strategy for the control of APEC infections in laying hens. Such studies should not only confirm its antibacterial efficacy but also evaluate its stability during digestive transit, bioavailability, safety, effects on the intestinal microbiota, and potential influence on egg production performance.

The assessment of the individual components of the plant-derived product demonstrated that all tested ingredients exhibited antibacterial activity against the APEC strains, although their potency varied. Cinnamon and oregano EOs exhibited the greatest antibacterial activity, both showing MICs values of 3900 µg/mL against all *E. coli* strains tested. These MICs values are consistent with previous studies reporting that cinnamon and oregano oils are among the most potent EOs against *E. coli*, with MICs generally ranging from 0.001 to 0.01 depending on bacterial strain and the extraction method [60, 61].

The strong antibacterial activity of cinnamon essential oil is primarily attributed to cinnamaldehyde, which represents up to 80% of the oil and has been shown to disrupt bacterial membranes, inhibit ATP synthesis and interfere with several essential metabolic bacterial enzymes [26]. Similarly, the antibacterial properties of oregano are generally associated with its high content of carvacrol and thymol, two phenolic monoterpenes known to increase membrane permeability, disrupt proton gradients, and promote leakage of intracellular constituents, leading to rapid bacterial death [62]. These findings suggest that cinnamon and oregano EOs may be the primary contributors to the antibacterial activity of the complete product used in the current study, while the other components may provide complementary effects.

In contrast, clove essential oil displayed a four-fold higher MIC than cinnamon and oregano oils MIC (15625 µg/mL), indicating lower antibacterial activity compared to these EOs. Nevertheless, this value remains within the range commonly reported in the literature for *E. coli* isolates [63]. The antibacterial activity of clove oil is largely attributed to eugenol, a phenolic compound capable of increasing membrane permeability, denaturing proteins and inducing oxidative stress in bacteria [64]. However, the outer membrane characteristic of Gram-negative bacteria limits the diffusion of eugenol, which may explain the comparatively lower efficacy observed in the present study relative to cinnamaldehyde and carvacrol [65]. In addition, differences in antibacterial activity among EOs are common and may be influenced by their chemical composition, the relative abundance of active constituents, and interactions between major and minor compounds [66]. On the other hand, pomegranate extract exhibited moderate antibacterial activity in the current study, with MIC values ranging from 10000 to 20000 µg/mL depending on the strain, although MIC values were slightly higher than those observed for cinnamon and oregano essential oils. This variability is expected because the antimicrobial efficacy of pomegranate depends strongly on the concentration of hydrolysable tannins, particularly punicalagin and ellagic acid, whose abundance varies according to fruit cultivar, extraction procedure and plant tissue used [27]. These compounds exert antibacterial effects through multiple complementary mechanisms, including membrane disruption, inhibition of extracellular enzymes, metal ion chelation and interference with bacterial biofilm formation [67]. Variability among bacterial isolates may additionally reflect differences in outer membrane permeability and stress-response mechanisms, both of which influence susceptibility to polyphenolic compounds [68]. The relatively consistent MIC values observed among the tested *E. coli* strains in this study further suggest that the antibacterial activity of pomegranate extract was not substantially influenced by differences in the AMR profiles of these strains.

The antibacterial interactions between EOs and pomegranate extract were evaluated using the FICI to determine whether combining these plant-derived ingredients could enhance their antibacterial activity against APEC strains. Overall, none of the tested combinations demonstrated a synergistic interaction under the experimental conditions used. Nevertheless, differences in FICI values were observed among combinations, suggesting that the nature and magnitude of interactions depended on the essential oil tested and the bacterial strain evaluated. This finding agrees with numerous studies demonstrating that synergistic interactions between natural compounds remain relatively uncommon and are highly dependent on bacterial strain, phytochemical composition and concentration ratios [69].

Among the three combinations investigated, the cinnamon–pomegranate combination exhibited the most favorable interaction profile. FICI values ranged from 0.8 to 1.8, with the lowest value observed for the ECL20719 strain (FICI = 0.8). According to the interpretation criteria used in the present study, these values indicate predominantly additive effects rather than true synergy. The relatively low FICI values obtained for this combination are consistent with the strong antibacterial activity individually demonstrated by cinnamon essential oil and pomegranate extract. Although studies investigating their combined activity remain limited, Yuan et al. reported enhanced antibacterial activity against *E. coli.* and *Staphylococcus aureus* when cinnamon essential oil and pomegranate peel extract were used together [70]. On the other hand, the clove–pomegranate combination produced intermediate FICI values ranging from 1.0 to 1.5, indicating additive to non-interactive effects. These results suggest that combining eugenol-rich clove essential oil with pomegranate extract neither substantially enhanced nor impaired antibacterial activity. A combination containing clove and pomegranate extracts, together with sage and Cassia fistula, showed activity against *E. coli* O157 [71]. However, the specific interaction between clove and pomegranate was not determined. Our findings therefore provide novel evidence regarding the combined antibacterial activity of these two plant-derived ingredients against APEC. Moreover, the oregano–pomegranate combination yielded the highest FICI values, reaching up to 3.0 for some APEC strains. These values indicate a non- interactive effect, suggesting that the combination did not provide a measurable advantage over the activity of the individual components. Previous studies have demonstrated the antibacterial activity of oregano essential oil and pomegranate extracts individually against a range of foodborne pathogens [72, 73]. However, to the best of our knowledge, the present study is the first to characterize the antibacterial interaction between these two plant-derived ingredients against clinical APEC strains using a combination-based approach (FICI).

Importantly, none of the tested combinations exhibited antagonism, indicating that the addition of pomegranate extract did not compromise the antibacterial activity of the EOs used in the tested commercial product. Several additional factors may also explain the limited interactions observed. In fact, the chemical composition of EOs varies according to plant origin, harvesting conditions and extraction methods, which can significantly influence antimicrobial activity [74]. Finally, the checkerboard method used in this study provides an endpoint assessment of bacterial growth inhibition and may therefore not fully capture the temporal dynamics of antimicrobial interactions, which can be better characterized using time-kill assays [75].

Interestingly, the predominance of additive interactions among O78:H9 strains is noteworthy, given that the ST23/O78 lineage is a well-described APEC lineage associated with phylogroup C [76]. However, the different interaction profiles observed among closely related strains suggest that ST and phylogenetic group alone may not fully explain the response to plant-derived combinations, highlighting the potential contribution of strain-specific genomic features[77]. In this context, the concordance between the H9 flagellar antigen and the additive phenotype observed in all O78:H9 strains raises the possibility that the flagellar antigen may contribute, at least in part, to the strain-dependent response to plant-derived combinations. Differences in flagellin structure among E. coli H-antigen types may influence flagellar properties and bacterial phenotypes, including motility and interactions with host cells. Moreover, plant-

derived compounds have been shown to affect flagellin expression and flagellum-mediated motility in E. coli, suggesting that variation in the flagellar system could potentially contribute to differences in bacterial responses to plant-derived compounds [78]. This hypothesis represents an interesting avenue for further investigation to better understand the mechanisms underlying the activity of plant-derived compounds against APEC strains. Nevertheless, these findings should be interpreted cautiously because only six APEC strains were included in the combination assays. Although the strains were intentionally selected to represent distinct AMR profiles and included strains with different molecular characteristics, this limited sample size substantially constrains the ability to establish robust associations between phylogenetic background and the observed interaction patterns. Studies involving a larger and more genetically diverse collection of APEC strains are needed to confirm these observations and identify genomic features associated with specific combination responses.

Conclusion

The present study provides new insights into the antibacterial potential of phytogenic products for targeting antimicrobial-resistant APEC strains associated with colibacillosis in laying hens. Despite the diversity of AMR determinants and phenotypic profiles among the APEC strains, the plant-derived product exhibited a uniform MIC under the standard culture or the complex matrix conditions, suggesting that its antibacterial activity was not substantially influenced by the AMR profiles represented in this APEC collection. All tested components of the plant-derived product exhibited antibacterial activity against the tested APEC strains, although their potency varied among the different ingredients. Cinnamon and oregano EOs demonstrated the strongest antibacterial activity. In addition, among the three combinations investigated, the cinnamon–pomegranate combination exhibited the most favorable interaction profile. These results provide a promising foundation for further research on this product as a complementary approach to antimicrobial therapy in the fight against APEC infections in laying hens. Future studies should validate its efficacy in an *in vivo* laying hen model of colibacillosis and assess its stability during gastrointestinal transit, bioavailability, effects on gut microbiota, and impact on laying performance and egg production.

Declarations

Credit authorship contribution statement

Angèle Eraud: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review and editing. **Alexander Thibodeau**: Methodology, Validation, Supervision, Writing – review and editing. **Marie-Lou Gaucher**: Methodology, Validation, Supervision, Writing – review and editing. **William Thériault**: Conceptualization, Formal Analysis, Project administration, Writing – review and editing. **Claire Carlu**: Methodology, Writing – review and editing. **Sandra Vaudaux**: Methodology, Writing – review and editing. **Mohamed Rhouma**: Conceptualization, Methodology, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review and editing. All authors read and agreed to the published version of the manuscript.

Data availability

The whole-genome sequencing data generated in this study have been deposited in the NCBI BioProject database under accession number PRJNA1531132. The corresponding raw sequencing data will be publicly available upon release by NCBI.

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Disclosures

C.C. and S.V. are employees of Phytosynthese, industrial partner supporting the present research project. Their involvement in the present study did not influence the study design, data analysis, interpretation of the results, or the decision to submit the manuscript for publication. All authors declare that they have no other competing financial or personal relationships that could have influenced the work reported in this article. Given his role as an Editor for Scientific Reports, MR had no involvement in the peer-review process for this manuscript and had no access to information regarding its peer review. Full responsibility for the editorial handling and peer-review process of this manuscript was delegated to another editor.

Declaration of generative AI use

Artificial intelligence (AI) (ChatGPT) was used solely to improve the language, grammar, and readability of the manuscript. All scientific content, study design, data analysis, interpretation of results, and conclusions were developed and verified by the authors, who take full responsibility for the content of this manuscript.

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Tables

Tables 1 to 6 are available in the Supplementary Files section.

Figures

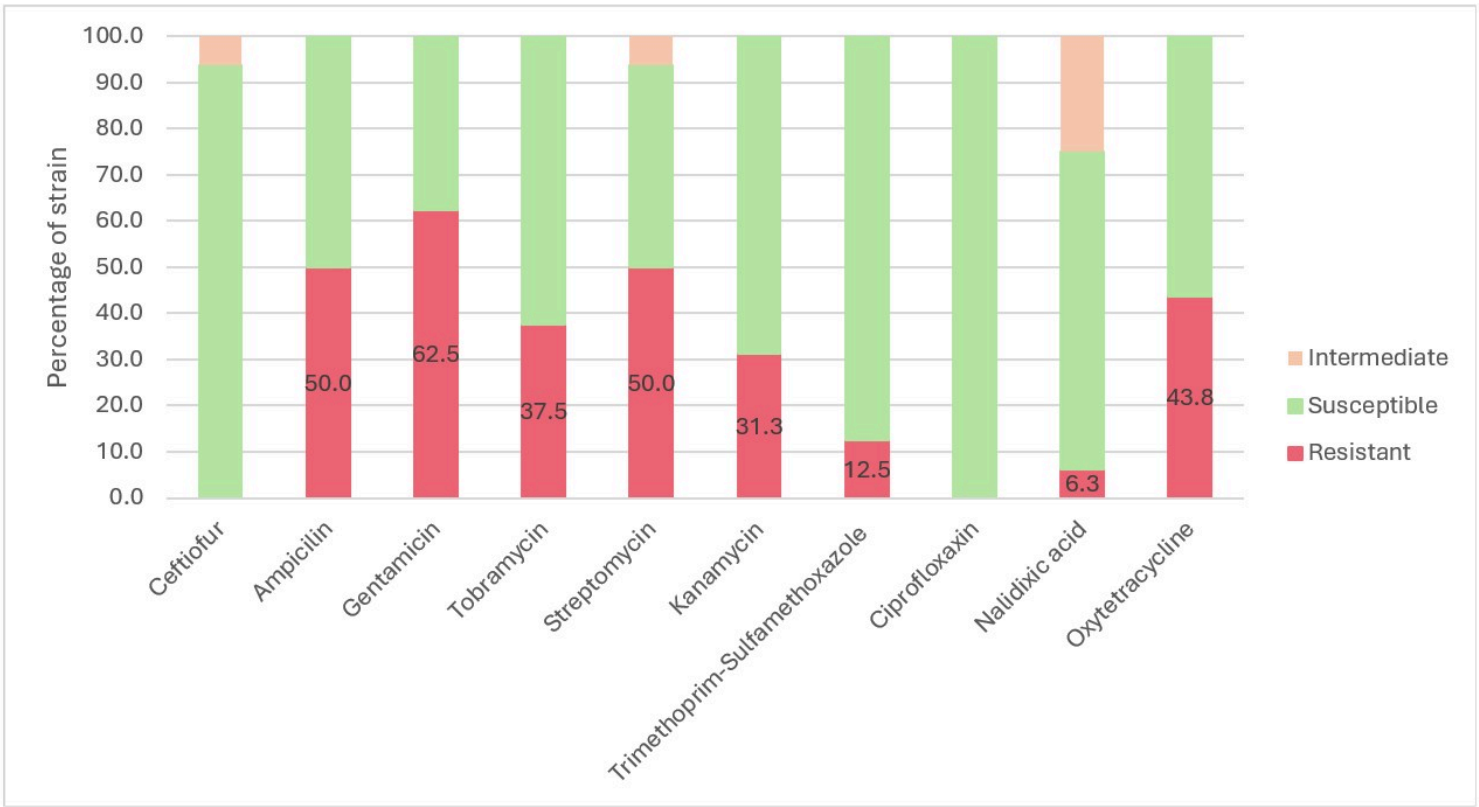


Figure 1

Percentage distribution of phenotypic antimicrobial susceptibility profiles among APEC strains (n = 16) for the antimicrobials tested.

Supplementary Files

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- Tables.docx
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