Original Research Article

Antimicrobial resistance profile and antimicrobial activity of the methanolic extract of *Pereskia aculeata* Mill. against *Escherichia coli* strains isolated from broiler chicken carcasses

ABSTRACT

Objective: To evaluate the antimicrobial resistance profile of *E. coli* strains isolated from broiler carcasses, and assess the antimicrobial activity of the methanolic extract of *Pereskia aculeata* Mill. by determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC).

Location and duration of the study: The study was conducted in a poultry slaughterhouse in Paraná, Brazil, and at the Laboratory of Preventive Veterinary Medicine and Public Health, Universidade Paranaense, from November 2022 to December 2023.

Methodology: Samples were collected by washing the carcasses from two broiler chicken farms, with 20 samples taken from each farm. The objective was to isolate enterobacteria and evaluate their antimicrobial resistance profiles using the disk diffusion method. Additionally, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the methanolic extract of *P. aculeata* Mill. were determined.

Results: All samples were contaminated with enterobacteria, and one strain of *E. coli* was isolated from each sample. No significant differences ($P > 0.05$) were found in the resistance profiles for the various antimicrobials evaluated when comparing poultry farms, A and B, with amoxicillin demonstrating the highest percentage of resistance. The multidrug resistance profile was higher in poultry farm B (30%) compared to poultry farm A (25%). Determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) was not possible, as all *E. coli* strains grew at all concentrations tested, which were less than or equal to 20 mg/mL. The compounds present in the extract at the highest concentrations were trans-cinnamic acid (50.13 mg/100 g) and caffeic acid (17.03 mg/100 g).

Conclusion: Broiler carcasses are carriers of *E. coli* strains with a multidrug-resistant profile to antimicrobials, and the methanolic extract of *P. aculeata* Mill. does not inhibit its growth in concentrations less than or equal to 20 mg/mL, demonstrating the need for new studies with other extraction forms.

Keywords: Birds; Minimum Bactericidal Concentration; Minimum Inhibitory Concentration; Multiresistance; Enterobacteriaceae; Ora-pro-nobis; Phytogenic.

1. INTRODUCTION

Brazil is the world's second-largest producer and largest exporter of chicken meat, with a production of 14.972 million tons in 2024 [1]. This growth can be attributed not only to the development of the poultry market but also to high population growth and urban expansion, which have led to an increasing search for foods that meet population demand and have considerable energy value. In this sense, chicken meat is a protein source with high nutritional value and an affordable cost, in addition to having a relatively fast production cycle [2]. This justifies the high per capita consumption, which in 2024 was 45.5 kilos per inhabitant [1].

On the other hand, there is a growing social concern regarding the use of antimicrobial agents in animal production. In poultry farming, antimicrobials have also been used as performance enhancers and as prophylactic and curative agents against various diseases [3], given their action against pathogenic agents through the competitive exclusion mechanism. However, this can lead to the selection of microbiota and the induction of resistance [4,5].

Antimicrobial resistance is defined as a characteristic that bacteria possess or acquire against agents to which they were previously considered sensitive, losing this sensitivity [6]. This resistance occurs naturally, intrinsically, or acquired, through the activation of genes upon exposure to antimicrobials, gene transfer between bacteria, or even through innate mechanisms and mutations [7].

The indiscriminate use of antimicrobials in the animal production chain can impact public health by contaminating food intended for both humans and animals with multiresistant microorganisms. In addition to the possibility of chemical residues in food of animal origin, there may be a widespread dissemination of resistant bacteria in the population [8].

Therefore, research is being conducted to find viable and effective alternatives to conventional antimicrobial agents. As an alternative to these, the use of beneficial microorganisms and their products, phytotherapeutic agents, and plant extracts has been described [4,9].

In this sense, we can mention the species *Pereskia aculeata* Mill., a cactus recognized for the high nutritional value of its leaves [10], as well as its antioxidant potential [11, 12, 13, 14]. However, there are few studies related to its antimicrobial capacity [15].

Souza *et al*. [14] evaluated the antimicrobial potential of two extracts (obtained using petroleum ether and chloroform as solvents) of *Pereskia aculeata* Mill. found that the extract in which petroleum ether was used inhibited the growth of *Escherichia coli*, one of the main pathogenic bacteria that causes major economic losses in the poultry industry [16] and is part of the enterobacteria, being considered a target of interest for microbiological analysis of broiler carcasses [17].

In addition, according to Ibrahim *et al*. [18], antimicrobial resistance in *E. coli* strains can be considered a public health concern, as they can be transmitted to humans through direct contact with birds or via the food chain, highlighting the importance of evaluating their presence in broiler carcasses.

Therefore, the objective of the present study was to evaluate the antimicrobial resistance profile and the antimicrobial activity of the methanolic extract of *Pereskia aculeata* Mill, against *E. coli* strains isolated from broiler carcasses from a slaughterhouse in the northwest region of Paraná.

2. MATERIAL AND METHODS

The present experiment was conducted in a poultry slaughterhouse in the northwest region of Paraná, Brazil, following approval by the Animal Use Ethics Committee (CEUA) of Universidade Paranaense under protocol 40065/2023.

The birds originated from two poultry farms, A and B, located on separate properties. From each source (poultry farm), 20 broiler carcasses were randomly selected before the stage of evisceration, totaling 40 carcasses, for collecting samples for counting and isolating enterobacteria.

The carcasses weighed about 3 kg and were placed in sterile bags containing 450 mL of peptone water, which was then shaken for approximately 30 seconds. A 100 mL aliquot of the sample was collected and transferred to another sterile bag, following the procedure described by Pacheco [19]. The samples were refrigerated and transported to the Laboratory of Preventive Veterinary Medicine and Public Health of the Postgraduate Program in Animal Science at Universidade Paranaense for processing.

2.1 Sample processing

In the laboratory, the samples were incubated for 24 hours in a microbiological incubator at 37 °C and then plated on MacConkey agar. They were incubated again for 24 hours at 37 °C. After

incubation, one of the predominant colonies was isolated for later identification. For conservation, all selected and isolated colonies were cultured in Brain Heart Infusion (BHI) medium and then stored in 80% glycerol at -20 °C.

2.2 Identification of Enterobacteria

To identify the enterobacteria, the isolates were cultured in BHI and incubated in a microbiological incubator for 24 hours at 37 °C. Subsequently, they were plated in MacConkey agar and incubated again under the same conditions to isolate pure colonies. The identification of enterobacteria was performed based on morphological and biochemical characteristics, as described by Quinn *et al*. [20]. For this, test tubes were used to perform the biochemical tests: LIA (Lysine Iron Agar), MIO (Motility, Indole, Ornithine), TSI (Triple Sugar Iron), Simmons Citrate, and Urea.

2.3 Phenotypic antimicrobial susceptibility tests

The agar disk diffusion method was used to assess the antimicrobial susceptibility profile, as recommended by the Clinical and Laboratory Standards Institute [21].

The inoculum was prepared by suspending isolated colonies selected from the MacConkey agar plate in BHI medium for 18-24 hours. The suspension was adjusted to a bacterial concentration corresponding to a turbidity of 0.5 on the McFarland scale, corresponding to approximately 1.5 × 10^8 CFU per mL [21]. Then, a swab was immersed in the suspension and inoculation was performed on the surface of the Müeller-Hinton agar plate by rubbing the swab across the entire sterile agar surface. The procedure was repeated twice more, rotating the plate approximately 60° each time to ensure uniform distribution of the inoculum [21].

13 antimicrobials from the following classes were tested: Penicillin's – amoxicillin 10 µg (AMO); 3rd and 4th generation cephalosporins: ceftiofur 30 µg (CTF), ceftazidime 30 µg (CAZ), cefotaxime 30 µg (CTX); Monobactams: aztreonam 30 µg (ATM); Carbapenems: imipenem 10 µg (IPM); Cephamycin's: cefoxitin 30 µg (CFO); Fluoroquinolones: enrofloxacin 5 µg (ENO), norfloxacin 10 µg (NOR) and ciprofloxacin 5 µg (CIP); Aminoglycosides: gentamicin 10 µg (GEN) and tobramycin 10 µg (TOB); Folate pathway inhibitors: sulfamethoxazole + trimethoprim 25 µg (SUT).

After 15 minutes of placing the discs, the plates were inverted and incubated at 37°C [21] and kept for an 18- to 24-hour incubation period. The halos were measured in millimeters using a caliper on the back of the inverted petri dish. The diameters of the inhibition halos were then interpreted and classified as sensitive, intermediate, or resistant, according to the criteria established by BrCAST [22], except for the antimicrobial enrofloxacin, which was classified according to the CLSI criteria [23]. All isolates classified as intermediate were classified as resistant.

2.4 Determination of the multidrug resistance profile

The bacterial isolates were classified according to the multidrug resistance (MDR) profile described by Magiorakos *et al*. [24]. MDR is defined as isolates that are not susceptible to at least one agent in more than three classes of antimicrobials. In addition, the Multiple Antimicrobial Resistance Index (MARI) was calculated according to the criterion established by Krumperman [25].

2.5 Obtaining the methanolic extract of *P. aculeata* Mill.

The leaves of Ora-pro-nóbis (*Pereskia aculeata* Mill.) were collected at the Medicinal Garden of the Universidade Paranaense, located at latitude 23°46'10.9"S and longitude 53°16'39.6"W, in the city of Umuarama, state of Paraná, Brazil, early in the morning, during the summer. The botanical material of Ora-pro-nóbis was deposited in the Herbarium of the Medicinal Garden under registration number 88 and registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge – SisGen under registration number AA63755. After collection, the leaves were selected, washed, and dried in a forced air oven at 35°C for 15 days. Subsequently, the material was crushed, and the particle size was determined to be 850 µm. The powder was subjected to dynamic maceration with solvent renewal with methanol. Then, the filtrate was concentrated under reduced pressure in a rotary evaporator (Tecnal TE-211 model) at 35°C, until the crude extract was obtained. Subsequently, the extract was frozen at -20°C until its use to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

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2.6 Analysis of the composition of some phenolic acids through High-Performance Liquid Chromatography (HPLC)

The extract was purified with 1 M barium hydroxide and 5% zinc sulfate solution, filtered (PVDF hydrophobic membrane, pore size 0.45 µm and 25 mm in diameter) and analyzed by high performance liquid chromatography HPLC 20A (Prominence, Shimadzu), consisting of a UV detector (SPD-20A, Shimadzu®), with wavelengths of 280 and 320 nm. A column oven (CTO-20A, Shimadzu®) maintained the C-18 column (Shim-pack CLC-ODS (H)™, 25 cm x 4.6 mm x 5 mm, Shimadzu) at 25 ºC. Using a manual injector (SIL-10A, Shimadzu®), 20 µL of the extract was added, and a quaternary pump (LC-20AT, Shimadzu®) was operated at a flow rate of 0.8 mL min-1.

For the chromatographic separation, ultrapure water acidified 0.05% with formic acid (A) and methanol acidified 0.1% with formic acid (B) were used as mobile phases in gradient elution mode: 0.01 to 5 minutes - 20% B, 5 to 25 minutes - 50% B, 25 to 30 minutes - 80% B. For quantification, solutions of gallic, coumaric, ferulic, caffeic, trans-cinnamic acids, and the flavonoids quercetin and kaempferol (1 mg mL−1 to 10 mg mL−1) were prepared to establish the calibration curves (R2>0.99), with the results expressed in mg 100 g-1 of sample [26].

2.7 Determination of the Minimum Inhibitory Concentration (MIC) of the methanolic extract of Ora-pro-nóbis (*Pereskia aculeata* Mill.)

As described in CLSI [27], the broth microdilution method was used with 96-well ELISA plates. The concentrations of 20, 10, 5, 2.50, 1.250, 0.625, 0.312, 0.156, 0.078, and 0.039 mg/mL were tested. These assays were performed in triplicate. After an incubation period of 24 hours at 37ºC, the plates were read to determine the MIC using 2,3,5 triphenyl tetrazolium chloride as a developer. The MIC was defined as the lowest concentration of the extract in mg/mL capable of preventing bacterial growth [28].

2.8 Determination of the Minimum Bactericidal Concentration (MBC) of the Methanolic Extract of Ora-pro-nóbis (*Pereskia aculeata* Mill.)

The Minimum Bactericidal Concentration (MBC) was determined by transferring a sample of the contents of each of the 96 wells of each plate to a Mueller Hinton Agar plate, with the aid of a replicator, and incubating at 36ºC for 24 hours. The MBC was determined by the absence of visible bacterial growth after incubation.

2.9 Statistical analysis

The absolute (n) and relative (%) frequencies of the resistance profile results for the different antimicrobials were determined. The Chi-square test compared differences in the resistance profile concerning the origin (avian) with Yates' correction or Fisher's exact test. Descriptive statistics were performed to determine the minimum value, maximum value, mean, and coefficient of variation for the results of the MARI calculation. These results were compared using the student's t-test for two independent samples. The MIC and MBC results were expressed as absolute values in mg/mL. A significance level of 5% was considered for all analyses. The analysis was performed using the Bioestat 5.3 program [29].

3. RESULTS AND DISCUSSION

Escherichia coli strain was isolated from each carcass sample collected in this study and tested for antimicrobial resistance. There were no differences (P>0.05) in the resistance profile for the different antimicrobials evaluated when comparing poultry farms A and B (Table 1). However, poultry farm B showed a higher percentage of resistance to the following antimicrobials: amoxicillin, ceftiofur, enrofloxacin, norfloxacin, ciprofloxacin, and sulfamethoxazole + trimethoprim, and poultry farm A showed resistance to cefotaxime, aztreonam, cefoxitin, and gentamicin. No isolate was resistant to imipenem and tobramycin.

Table 1 – Antimicrobial resistance profile of *Escherichia coli* strains isolated from broiler carcasses from two poultry farms in an integrated poultry farm located in the northwest region of the state of Paraná

Classes	Antimicrobial	n, %		P-value
		Poultry Farm A (n=20)	Poultry Farm B (n=20)	
Penicillins	Amoxicillin	14 (70%)	15 (75%)	1,000*
3rd and 4th Generation Cephalosporins	Ceftiofur	7 (35%)	8 (40%)	1,000*
	Ceftazidime	4 (20%)	4 (20%)	0,3416**
	Cefotaxime	7 (35%)	6 (30%)	0,7301*
Monobactams	Aztreonam	8 (40%)	7 (35%)	0,3006*
Carbapenems	Imipenem	0 (0%)	0 (0%)	---
Cephamycins	Cefoxitin	1 (5%)	0 (0%)	1,000**
Fluoroquinolones	Enrofloxacin	11 (55%)	14 (70%)	1,000**
	Norfloxacin	5 (25%)	9 (45%)	0,3200*
	Ciprofloxacin	5 (25%)	7 (35%)	---
Aminoglycosides	Gentamicin	7 (35%)	3 (15%)	0,1818**
	Tobramycin	0 (0%)	0 (0%)	---
Folate pathway inhibitors	Sulfamethoxazole + Trimethoprim	4 (20%)	9 (45%)	0,1796*

*Chi-square with Yates correction; ** Fisher's exact

Cardoso *et al*. [30] evaluated the antimicrobial resistance profile of *E. coli* strains isolated from fragments of commercial poultry organs, including broiler breeders, laying hens, and broiler chickens. They found that amoxicillin showed the highest resistance (82.8%). This result is like the findings in the present study, where 70% and 75% resistance to amoxicillin was observed in *E. coli* isolates from broiler carcasses. Similarly, Bortoli *et al.* [31] collected liver, spleen, and heart from broiler chickens from five farms and found that 93.3% of the *E. coli* isolates were resistant to amoxicillin.

In a study conducted in Ecuador, Ortega-Paredes *et al.* [32] evaluated the resistance profiles of *E. coli* strains isolated from broiler carcasses (breast skin) from various markets. They found that 100% of the isolates were resistant to several beta-lactam antimicrobials, including ampicillin, cephalothin, ceftriaxone, cefuroxime, and cefotaxime.

In a study conducted in China, Wu *et al*. [33] found that *E. coli* isolates from broiler carcasses exhibited a notably high resistance to several antimicrobials, including amoxicillin combined with clavulanate, with a resistance rate of 99.2%. The findings, along with existing literature, indicate that the presence of *E. coli* strains with varying resistance profiles depends on the sample. However, chicken products are a potential source of multidrug-resistant *E. coli*, posing a significant public health concern.

Regarding the Multiple Antimicrobial Resistance Index (MARI), no significant differences were observed between the different poultry houses (P> 0.05). Nevertheless, poultry house B showed a higher average MARI of 0.315 (see Table 2).

Table 2 - Multiple Antimicrobial Resistance Index (MARI) of *Escherichia coli* strains isolated from broiler carcasses from two poultry farms in an Integration located in the northwest region of the State of Paraná

Descriptive statistics	Poultry Farm A	Poultry Farm B
Minimum	0	0
Mean*	0,265	0,315
Maximum	0,615	0,692
Coefficient of variation	68,7%	68,0%

Not significant by the T-test for two independent samples

According to Krumperman (1983) [25], the choice of MARI 0.2 to differentiate between isolates with high or low risk of contamination is arbitrary, since it depends on each situation. This is due to isolates with an average MARI of 0.2 may also represent contamination of passing concern. In the present study, comparing the multidrug resistance index according to the criteria of Magiorakos *et al.* [24], it was found that Aviary B also presented a higher percentage of samples with a multidrug resistance profile (30%) vs. 25% (Aviary A). The number of classes in which the isolates were non-susceptible was four for Aviary A and four, five, and six for Aviary B. It is worth noting that isolates with

MARI 0.385 were found to be classified as multidrug resistant or not according to the criteria of Magiorakos *et al*. [24].

Regarding the Minimum Inhibitory Concentration (MIC) of the methanolic extract of *P. aculeata*, it was found that 100% of the *E. coli* isolates evaluated were not inhibited at the concentrations evaluated, i.e., it was impossible to determine the MIC. Similarly, it was impossible to determine the Minimum Bactericidal Concentration (MBC), since all isolates grew at all concentrations of the methanolic extract, i.e., between 20 and 0.039 mg/mL.

Mezalira *et al*. [34] determined the MIC of the aqueous extract of *P. aculeata*. They found that only eight *E. coli* isolates (8%) from cloacal swab samples of broiler breeders, nest eggs, and floor eggs from two breeders could be used to determine the MIC, which was 12 mg/mL.

It is also worth noting that, according to the classification provided by Pandini *et al*. [35] regarding the level of inhibition acceptable for characterizing the viability or otherwise of plant extracts, the MIC of 12 mg/Ml.

Colacite *et al*. [36] evaluated the antimicrobial activity of ethanolic and methanolic extracts of *Pereskia aculeata* against the bacterium *E. coli*, but did not find any inhibition of its growth. On the other hand, the methanolic extract inhibited the bacterium *Klebsiella pneumoniae*, demonstrating variation in relation to the bacterium evaluated.

In contrast, Belo *et al.* [37] evaluated the essential oil of *P. aculeata*. They found inhibition of *K. pneumoniae* and *Staphylococcus aureus* at a concentration of 90%, but did not achieve the same results at lower concentrations.

The variations in the MIC results in different studies may be related to several factors, including the microorganism and strain evaluated, the origin and time of harvest of the plant material, the method of preparation of the extract (fresh or dry material), as well as differences in the species of the plant evaluated [38].

The species of origin and the time of harvest of the plant material influence the concentration of the different bioactive compounds [39]. In the present study, a qualitative evaluation of the bioactive compounds presents in the methanolic extract of *P. aculeata* was not performed. The quantitative analysis of some phenolic acids demonstrated the highest average for trans-cinnamic acid, followed by caffeic and chlorogenic acids (Table 3).

Table 3 - Mean ± standard deviation of the concentration (mg/100g) of gallic acid, trans-cinnamic acid, chlorogenic acid, caffeic acid, coumaric acid, ferulic acid, quercetin, kaempferol, and catechin in the methanolic extract of *Pereskia aculeata*.

Compounds	**Mean ± standard deviation (mg/100g)**
Gallic acid	5,11±1,53
Trans-cinnamic acid	50,13±0,13
Chlorogenic acid	8,08±0,05
Caffeic acid	17,03±0,09
Coumaric acid	2,67±0,02
Ferulic acid	4,28±0,31
Quercetin	1,07±0,07
Kaempferol	2,39±0,05
Catechin	3,70±0,29

Most studies perform qualitative analysis of the bioactive compounds present in plants. Morais *et al*. [40] evaluated the phytochemical profile of the aqueous extract of fruits and flowers of *P. aculeata*. Also, they detected the presence of gallic acid, caffeic acid, coumaric acid, ferulic acid, quercetin, kaempferol, among others.

Similarly, Garcia *et al.* [41] detected the presence of caftaric acid, quercetin-3-O-rutinoside, and isorhamnetin-O-pentoside-O-rutinoside in the hydroethanolic extract of *P. aculeata*, demonstrating variations in the presence of compounds according to the extraction method. Studies conducted by Fu *et al* [42] demonstrate that caffeic acid at concentrations of 7.95, 6.25, 3.89, 1.18, 3.12, and 15.5 (mg/mL) presents inhibitory activity against strains of *E. coli* and *S. aureus*.

4. CONCLUSION

It has been concluded that broiler carcasses carry *E. coli* strains with a multi-resistant profile to antimicrobials. Additionally, the methanolic extract of *P. aculeata* Mill does not inhibit the growth of these

strains at concentrations of 20 mg/mL or lower. This finding highlights the necessity for further studies using different extraction methods or evaluating higher concentrations of the extract.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

ETHICAL APPROVAL

The Ethics Committee previously approved the work for the Use of Animals (CEUA) of Universidade Paranaense under protocol 40065/2023.

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