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Molecular Identification of Bacteria and Fungi Isolated from Chicken Parts in Poultry Farm in Port Harcourt Metropolis

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ABSTRACT

Contamination of poultry products with pathogenic bacteria and fungi poses significant public health risks and economic losses. This study aimed to molecularly identify and detect resistance genes in bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Campylobacter* spp.) and fungi (*Fusarium spp.*, *Aspergillus flavus*, *Penicillium spp.*) isolated from samples of chicken parts (breast, gizzard, and cloacae) obtained from three (3) poultry farms. Total heterotrophic bacteria count was 3.36×10^6 CFU/g. Total occurrence of the isolates in all the chicken parts were; *Escherichia coli* 32×10^6CFU/g, *Staphylococcus aureus* 30×10^6CFU/g, *Campylobacter* 25×10^6CFU/g. Total fungi was 26×10^4CFU/g, *Fusarium* sp 23×10^3CFU/g, *Penicillium* sp 19×10^3CFU/g, *Aspergillus flavus* 16×10^3CFU/g. DNA was extracted from the isolates and analyzed using polymerase chain reaction (PCR) with species-specific primers targeting the 16S rRNA gene for bacteria and ITS regions for fungi. Molecular characterization identified the isolates as; *Escherichia coli* AM944637.2, *Staphylococcus aureus* AP017923.1, *Campylobacter upsaliensis* AB980278.1, *Fusarium solani* EU329657.1, *Aspergillus flavus* MT594359.1 and *Penicillium oxalicum* MK389646.1. The detection of antibiotic resistance gene and Agarose Gel Electrophoresis image showing amplification of ESBL (OXA-48) gene at 450bp revealed that, *Staphylococcus aureus* and *Campylobacter upsaliensis* showed positive amplification meaning that the gene is present in the isolates. While the amplification of antibiotic resistance gene and Quinolones gene (qnrB) at 250bp were positive to *Escherichia coli* and *Campylobacter upsaliensis*. This study highlights the potential risks associated with poultry contamination and emphasizes the need for stringent monitoring and control measures in the indiscriminate use of antibiotics in poultry production and processing.

Keywords: Molecular Identification, Chicken Parts, Poultry Farm, DNA Extraction, Antibiotic Resistance Genes.

Introduction

Poultry products serve as a primary source of animal protein worldwide and are integral to meeting the nutritional demands of a growing population. However, they are frequently associated with the carriage of pathogenic microorganisms, raising significant public health concerns (Mead, 1999; Havelaar *et al*., 2015). Among the bacterial pathogens, *Escherichia coli*, *Staphylococcus aureus*, and *Campylobacter spp.* have been identified as major contributors to foodborne illnesses, antimicrobial resistance, and zoonotic diseases (Kaakoush *et al.,* 2015; Farooq *et al*., 2019). Similarly, fungal contaminants such as *Fusarium* spp., *Aspergillus flavus*, and *Penicillium* species are known for their potential to produce harmful mycotoxins, which pose risks to food safety and human health (Pitt & Hocking, 2009; Marin *et al*., 2013).

The detection and accurate identification of these microorganisms are critical for ensuring food safety and mitigating the associated public health risks. While traditional culture-based techniques have been widely employed, they are often time-consuming and may lack the precision required for species-level identification (Janda & Abbott, 2007). In recent years, molecular methods such as polymerase chain reaction (PCR) and DNA sequencing have emerged as reliable tools for the rapid and specific detection of pathogens, offering higher sensitivity and specificity compared to conventional techniques (Amann *et al*., 1995).

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This study aimed to molecularly identify bacterial and fungal pathogens isolated from chicken parts samples using advanced molecular techniques. Specifically, the 16S rRNA gene was targeted for bacterial identification, while the internal transcribed spacer (ITS) regions were used to identify fungal isolates. By characterizing the microbial contaminants present in poultry products, this research seeks to highlight the prevalence of these pathogens and underscore the need for stringent monitoring and control measures in poultry production and processing.

Materials and Methods

Study Area

The study was carried out using chicken from Di Life Poultry Farm Seltex road, Gbalajam Woji, Mechis Farm at 59 Ogbunabali road, Ngozi and Ibeka farm at 30 SARS road, timber by NNPC filling station, Obio Akpor Local Government Area, Port-Harcourt, Rivers State.

Sample Collection

A total of 152 poultry product samples comprising of (water, feed, air faeces, meat, cloaca, gizzard and egg) were aseptically and randomly collected from the three different poultry farm location using a sterile swab stick. Samples were collected monthly for a period of three (3) months (May, June and July, 2024).

The samples were collected and packaged in a sterile transparent ziplock polypropylene bags containing ice block and were quickly transported to the microbiology laboratory within 1 hour of collection, and analysis was conducted within 3 hours of sample collection.

Serial Dilution and Incubation

For analysis, 10g of each of the poultry products sampled were macerated using a sterile marble mortar and pestle and were homogenized in 90mL sterile normal saline in sterile test tubes. The bottles were shaken thoroughly, ten (10) fold serial dilutions of the resultant homogenates were made to obtained 10^{-2} to 10^{-4} and 10^{-2} to 10^{-3} dilutions respectively.

Isolation of Bacterial Pathogens

For the isolation of bacterial pathogens, aliquots of the sample suspension were inoculated onto selective and differential media: Aliquot (0.1ml) were streaked onto Eosin Methylene Blue (EMB) agar and incubated at 37°C for 24 hours for the isolation of *E. coli*. Colonies with a characteristic metallic green sheen were selected for further analysis. Aliquot (0.1ml) were streaked onto Mannitol Salt Agar (MSA) and incubated at 37°C for 24 hours. Yellow colonies indicative of mannitol fermentation were identified as presumptive *Staphylococcus aureus*. Aliquot (0.1ml) were streaked onto Campylobacter Blood-Free Selective Agar Base supplemented with antibiotics (e.g., cefoperazone and amphotericin B) and incubated under microaerophilic conditions at 42°C for 48 hours for the isolation of *Campylobacter* spp. Presumptive colonies were further purified by subculturing on non-selective media for subsequent molecular identification (Holt, 1977; Cheesbrough, 2010).

Isolation of Fungal Pathogens

For the isolation of fungal species, aliquots of the sample suspension were inoculated onto Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol to suppress bacterial growth. Plates were incubated at 25°C for 5–7 days to promote fungal growth. The following methods were used for identifying fungal isolates:

Fusarium spp colonies were identified based on their cottony texture and pigmentation. *Aspergillus flavus* colonies with yellow-green pigmentation were noted, followed by microscopic examination to confirm the presence of vesicles bearing conidiophores. *Penicillium* spp. had velvety colonies with shades of green or blue were identified, and morphological features were confirmed microscopically (Barnett and Hunter, 1998).

Preservation of Isolates

All bacterial and fungal isolates were stored at -80°C in cryopreservation medium, e.g., glycerol stock or sterile saline for long-term storage and subsequent molecular analysis.

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Molecular Identification of Bacterial Isolates

DNA Extraction

The ZR Bacterial DNA Miniprep (Manufactured by Zymo Research) was used in the extraction of the bacterial DNA. In this method, two millilitres of the bacterial cell in broth were transferred into a ZR BashingTM Lysis Tube. In the tube, 750µl lysis solution was added. The solution was secured in a bead fitted with 2ml tube holder assembly and processed at a maximum speed for 5 minutes. The contents in the ZR BashingTM Lysis Tube were centrifuged in a microcentrifuge at 10, 000xg for 1 minute. After which, 400 µl supernatant was transferred to a Zymo-SpinTM IV Spin Filter (orange top) in a Collection Tube and centrifuged at 7,000 x g for 1 minute (Fontana *et al.,* 2005).

A volume of 1,200 µl Fungal/Bacterial DNA Binding Buffer was added to the filtrate in the Collection Tube and 800 µl of the mixture was transferred to a Zymo-SpinTM IIC Column in a Collection Tube and centrifuge at 10,000 x g for 1 minute. The flow was discarded from the collection tube and the previous steps were repeated. After which 200 ul DNA Pre-Washed Buffer was added to the Zymo-Spin TM IIC Column in new Collection Tube and centrifuged at 10,000 x g for 1 minute. A volume of 500 ul Fungal/Bacterial DNA Wash Buffer was added to the Zymo-SpinTM IIC Column and centrifuged at 10,000 x g for 1 minute and the Zymo-SpinTM IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and 100ul (35 ul minimum) DNA Elution Buffer was added directly to the column matrix. This was later Centrifuged at 10,000 x g for 30 seconds to elute the DNA (Fontana *et al.,* 2005).

Amplification of 16S rRNA

The isolates' 16S rRNA region was amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microliters for 35 cycles. The PCR mixture contained the X2 Dream Taq Master mix from Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), primers at a concentration of 0.4M, and extracted DNA as a template. The PCR settings included initial denaturation at 95ºC for 5 minutes, denaturation at 95ºC for 30 seconds, annealing at 52ºC for 30 seconds, extension at 72ºC for 30 seconds for 35 cycles, and final extension at 72ºC for 5 minutes.

The result was resolved on a 1% agarose gel at 120 volts for 15 minutes and visualised using a UV transilluminator (Fontana *et al.,* 2005).

DNA Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of BigDye terminator v3.1 cycle sequencing kit. Bio- Edit software and MEGA 6 were used for all genetic analysis (Cheesbrough, 2010).

Phylogenetic Analysis

Similar sequences were downloaded from the National Centre for Biotechnology Information (NCBI) database using BLASTN, and the obtained sequences were edited using the bioinformatics tool trace edit. MAFFT was used to align these sequences. MEGA 6.0's Neighbor-Joining approach was used to infer the evolutionary history.

The bootstrap consensus tree derived from 500 replicates (Felsenstein, 1985) is used to illustrate the evolutionary history of the taxa studied. The evolutionary distances were calculated using the Jukes-Cantor technique (Jukes and Cantor, 1969).

Detection of resistant gene

Genomic DNA of antimicrobial resistance (AMR) genes of the isolates was determined using PCR amplification of the antibiotic resistance genes. The TEM gene was amplified using the SHV F: 5' CGCCTGTGTATTATCTCCCT-3' and SHV R: 5' CGAGTAGTCCACCAGATCCT-3' primers, SHV genes from the isolates were amplified on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 µl for 35 cycles.

The resultant product was resolved on a 1 % agarose gel at 120V for 25 minutes and visualized on a UV trans-illuminator for a 281 bp product size. Sizes of resolved products were 960 bp for the TEM gene (Fontana *et al.,* 2005).

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Results

The six microbial isolates had 100% similarity with the different isolates stored in the NCBI gene bank. An agarose gel electrophoresis of the amplified 16S rRNA gene of the three bacteria isolated and identified in this study is shown on Plates 1, along with a phylogenetic tree that illustrates the evolutionary distances between the bacterial isolates and the percentage of relatedness with their close relatives in the gene bank (Figure 1). All bacterial species share the same 16SrRNA gene.

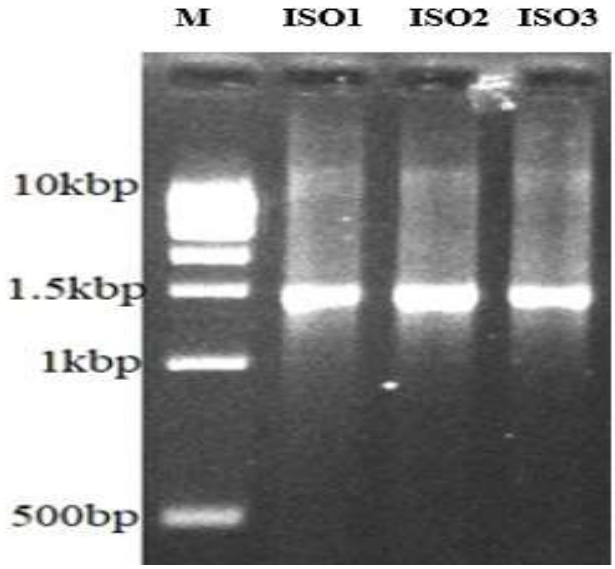

Plate 1: Agarose Gel Electrophoresis of Amplified 16srRNA gene of Bacterial Isolates (Lane M = Marker, ISO1= *Escherichia coli*, ISO2= *Campylobacter upsaliensis*, ISO3= *Staph. aureus*)

An agarose gel electrophoresis of amplified 16S rRNA gene of the three fungal isolated identified in this study is shown on Plate 2, along with a phylogenetic tree that illustrates the evolutionary distances between the fungal isolates and the percentage of relatedness with their close relatives in the gene bank (Figure 2).

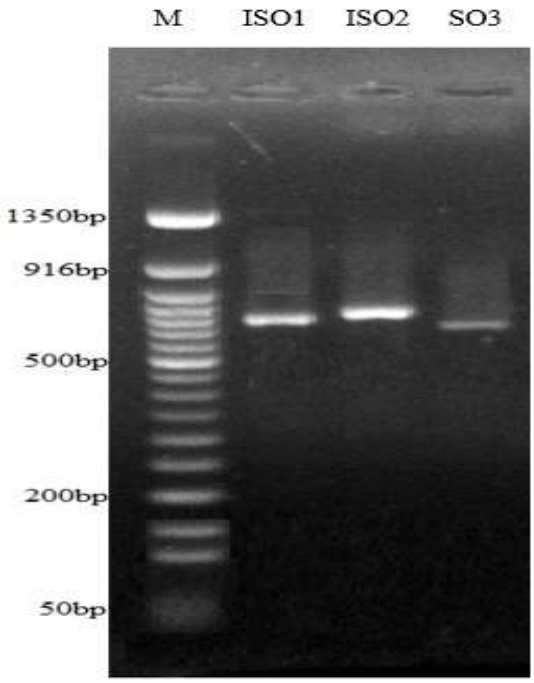

Plate 2: Agarose Gel Electrophoresis of Amplified internal transcribed spacer (ITS) gene at 650bp for fungal Isolates. Lane M = 50bp DNA ladder; ISO1= *Fusarium solani*, ISO2= *Aspergillus flavus*, ISO3= *Penicillium oxalicum*

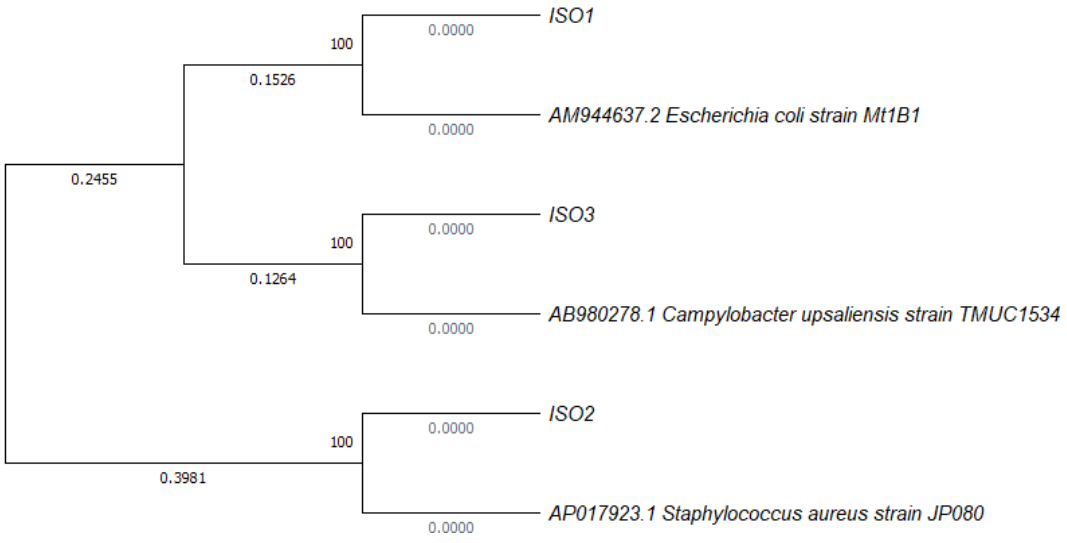

Fig. 1: Evolutionary relationships Between Bacterial Isolated from chicken parts

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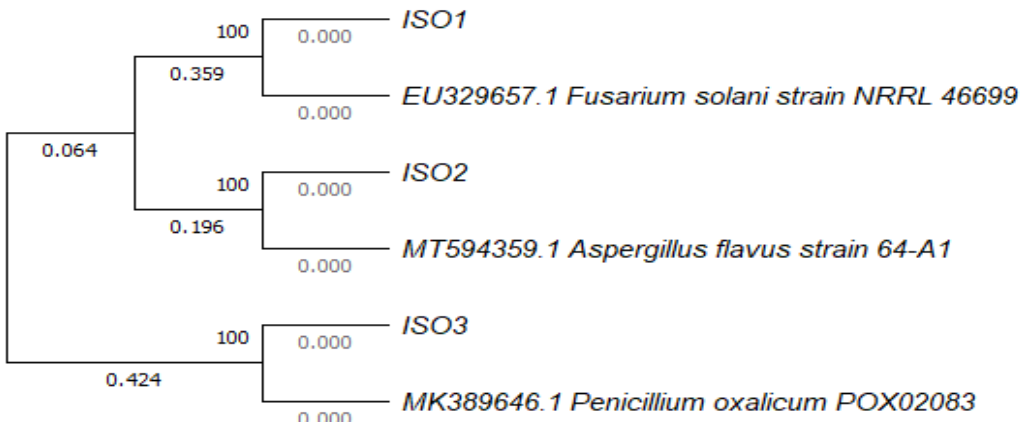

Fig. 2: Evolutionary relationships between fungi Isolated from chicken parts

Table 1: Phenotypic and Molecular Characteristics of Selected Microbial isolates from Samples Analyzed

Isolate code	Phenotype	NCBI gene bank	Percentage (%) similarity	Accession number
ISO1	*Escherichia coli*	*Escherichia coli* strain Mt1B1	100	AM944637.2
ISO2	*Staphylococcus aureus*	*Staphylococcus aureus* strain JP080	100	AP017923.1
ISO3	*Campylobacter* sp	*Campylobacter upsaliensis* strain TMUC1534	100	AB980278.1
ISO1	*Fusarium* sp	*Fusarium solani* NRRL 46699	100	EU329657.1
ISO2	*Aspergillus* sp	*Aspergillus flavus* 64-A1	100	MT594359.1
ISO3	*Penicillium* sp	*Penicillium oxalicum* POX02083	100	MK389646.1

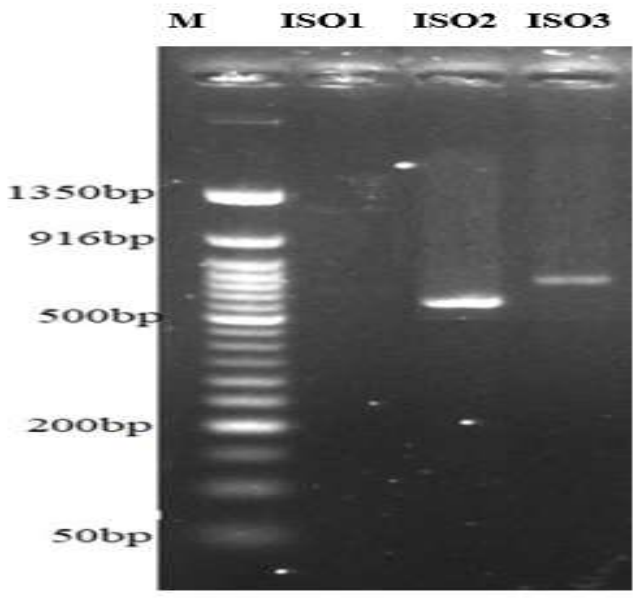

Plate 3: Gel image showing amplification of ESBL (OXA-48) at 550bp to 650bp. (Lane M = Molecular ladder; ISO1 =*E. coli*, ISO2 = *Staphylococcus aureus*, ISO3 = *Campylobacter upsaliensis*)

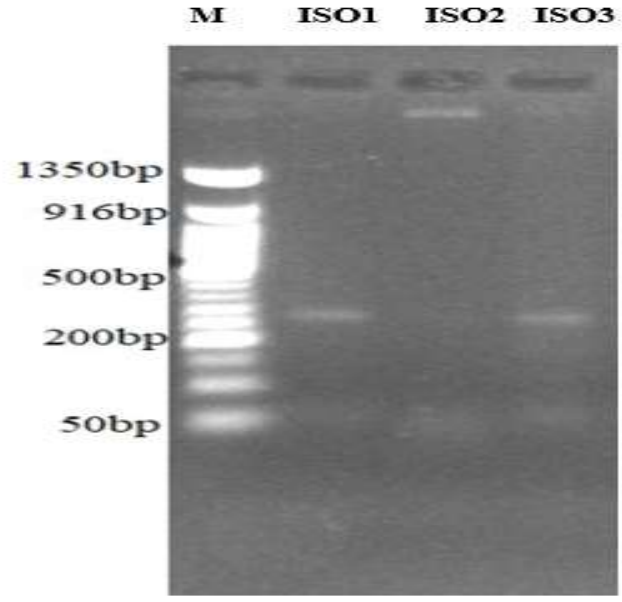

Plate 2: Gel image showing amplification of quinolones gene (qnrB) at 250bp (Lane M = Molecular ladder; ISO1 =*E. coli*, ISO2 = *Staph aureus*, ISO 3 = *Campylobacter upsaliensis*)

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Discussion

This study identified and characterized six microbial isolates from poultry samples, comprising three bacterial species (*Escherichia coli*, *Staphylococcus aureus*, and *Campylobacter* spp.) and three fungal species (*Fusarium* spp., *Aspergillus flavus*, and *Penicillium* spp.). The results showed that the 16S rRNA gene sequences of the bacterial isolates and the internal transcribed spacer (ITS) gene sequences of the fungal isolates exhibited 100% similarity with their closest relatives in the NCBI GenBank, confirming accurate identification.

The microbial isolates which were identified through molecular characterization and identification (Plate 1 and Plate 2), thus confirmed the identification of these isolates; *Escherichia coli* AM944637.2, *Staphylococcus aureus* AP017923.1, *Campylobacter upsaliensis* AB980278.1, *Fusarium solani* EU329657.1, *Aspergillus flavus* MT594359.1 and *Penicillium oxalicum* MK389646.1 according to 16S rRNA gene sequencing for the identification of microbial species. All the isolates identified through molecular characterization and identification showed that they all possess the exact similarities from the obtained 16S rRNA sequence of the isolates produced during the mega blast search were very similar to the sequences from the NCBI gene bank and all had 100% similarity index. An agarose gel electrophoresis amplified 16s rRNA gene of the five bacteria isolated and identified in this study. Phylogenetic analysis of 16S rRNA sequences of the microbial isolates showed distinct clustering and each phylogenetic tree had the same respective nodes showing that they evolved from the same ancestor. The evolutionary history was inferred using the Neighbor-Joining method with an optimal tree (Saitou and Nei, 2009), which helps in identifying closely related bacteria and their evolutionary, classify bacteria into distinct species or groups, study the spread of antibiotic resistance or virulence factors and also developed targeted treatments or prevention strategies. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown below the branches (Tamura *et al.*, 2004). The evolutionary distances were computed using the Jukes-Cantor method (Kumar *et al.*, 2018) and are in the units of the number of base substitutions per site.

This analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+ Non-coding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1953 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Kumar *et al.*, 2018). Through the evolutionary relationship of bacteria isolates in the study showed their phylogeny, genetic similarity and distinct characteristics.

The findings of this study underscore the critical need for effective monitoring and control measures to prevent the contamination of poultry products with both bacterial and fungal pathogens. The high similarity of the bacterial isolates to known pathogens highlights the importance of using molecular techniques for accurate and rapid identification of foodborne microorganisms.

Moreover, the detection of antibiotic resistance genes in *E. coli* and *S. aureus* isolates is a cause for concern, as it suggests the potential for the spread of resistant strains from poultry to humans, especially through the consumption of contaminated poultry products (Hernandez *et al*., 2005).

Similarly, the identification of mycotoxigenic fungi emphasizes the need for improved control measures in poultry farming to mitigate the risk of mycotoxin exposure. It is crucial to implement strategies that limit fungal contamination, such as proper feed storage, adequate drying techniques, and regular screening for fungal contamination in poultry products (Pitt & Hocking, 2009).

A study similar to this used 16S rRNA sequencing to identify bacterial contamination in retail poultry meat. And found high prevalence of *Escherichia coli* and *Staphylococcus aureus*, which aligns with this study. PCR-based detection of *Campylobacter jejuni* in broiler chickens were performed and a widespread of contamination was reported across farms, highlighting food safety risks. These findings support the use of 16SrRNA gene amplification for rapid detection of *Escherichia coli*, *Staphylococcus aureus*, and *Campylobacter* spp., as observed in the present study (Nordmann *et al*., 2012; Zhao *et al*., 2020; Salehi *et al*., 2018).

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Conclusion

This study provides valuable insights into the microbial contamination of poultry products, highlighting the presence of both bacterial and fungal pathogens. The molecular identification techniques employed were highly effective in accurately identifying the pathogens and gene profiling thereby providing essential data on their genetic characteristics. These findings emphasize the importance of regular surveillance and the implementation of effective control measures in poultry production to ensure food safety and reduce the risk of zoonotic transmission and antibiotic resistance.

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