**Antibiotic susceptibility profiles of *Escherichia coli* isolated from bird nests indicate that they can serve as reservoirs for antibiotic-resistant bacterial strains in the environment**

**Abstract**
The emergence and dissemination of antibiotic-resistant bacterial strains in environmental niches is a growing public health concern with significant implications for human health. Wild birds can potentially transmit antibiotic-resistant bacteria across ecosystems, and their frequent nesting near human habitats heightens the risk of human exposure. This study evaluated the antibiotic susceptibility profiles of Escherichia coli strains isolated from bird nests collected from various locations in Akoko Southwest, Ondo State, Nigeria. *E. coli* was isolated from the collected bird nests by inoculation on Eosin Methylene Blue (EMB) agar, and the identity of the isolates was confirmed based on their cultural characteristics, cellular morphology, and selected biochemical reactions. The phenotypic susceptibility of the *E. coli* isolates to selected antibiotics was assessed using the disc diffusion assay. *E. coli* was isolated from 53.13% of the collected nests, with concentrations ranging from 2.0x10¹ to 2.8x10² CFU/g. The *E. coli* strains isolated from the bird nests in this study exhibited resistance to several of the antibiotics tested, with resistance predominantly observed for tetracycline (41.18%) and amoxicillin-clavulanate (47.06%). Results from this study demonstrated the significance of bird nests as reservoirs for antibiotic-resistant bacterial strains in the environment.

Keywords: *Escherichia coli*, Antibiotic resistance, Bird nests, Antimicrobial Resistance (AMR)

**Introduction**
Infections caused by antibiotic-resistant pathogenic bacterial strains constitute a major threat to public health globally, and efforts aimed at understanding their emergence and distribution remain a top priority. For instance, an estimated 4·71 million deaths globally were associated with antibiotic resistance in 2021 (Naghavi et al., 2024). Given the high burden of antibiotic resistance, it’s important to fully understand the sources and distribution of antibiotic-resistant bacterial strains. While a great deal of effort has been dedicated to understanding the emergence and distribution of antibiotic-resistant bacterial strains in clinical and agricultural settings, the emergence and distribution of antibiotic-resistant strains in natural environments has been less exhaustively investigated (Fletcher, 2015). Niches in the natural environment are also important reservoirs for antibiotic-resistant bacterial strains from which humans can become infected directly or indirectly through vectors or inanimate fomites (Da Costa et al., 2013). Therefore, it is important to understand the contribution of environmental factors in the spread of antimicrobial resistance.

Animals such as free-living birds have a very good potential to disseminate microorganisms in environmental niches. Free-living birds can interact with diverse niches in the natural and built environment, thus have the potential to disseminate microorganisms in a wide range of environmental niches. Additionally, their ability to travel long distances enables them to disseminate microbial species over a wide range of environmental niches. Free-living birds are increasingly being recognised as an important source of human pathogenic antimicrobial-resistant strains in environmental niches (Kobuszewska and Wysok, 2024). Free-living birds have been linked to the transmission of important human pathogens such as human pathogenic strains of *Escherichia coli, Salmonella* and *Campylobacter* species (Smith et al., 2020; Batista et al., 2022).

*E. coli* is commonly used to monitor antibiotic resistance in the environment due to its wide distribution and ability to persist wide range of conditions in the natural environment (Anjum et al., 2021). Additionally, the potential for *E. coli* strains to acquire antibiotic resistance through a wide range of mechanisms makes it an ideal organism to monitor the emergence of antibiotic resistance in environmental niches. Also, pathogenic *E. coli* strains are important human and animal pathogens which can acquire antibiotic resistance from non-pathogenic strains, therefore monitoring antibiotic resistance in *E. coli* environmental strains will provide important information on the pool of antibiotic resistance genes in environmental niches (Ramos *et al*., 2020; Nyirabahizi *et al*., 2020).

Bird nests are unique environmental niches where bacterial species can persist, interact and exchange genetic materials (Xin et al., 2023). The relatively stable environmental conditions in bird nests and the availability of organic materials from bird droppings, nesting materials and food scraps support microbial growth in bird nests (Zabłotni et al., 2020). The microbial population in bird nests can thus reach a density that can enhance the exchange of genetic materials between different microbial species and may result in the spread of antibiotic resistance or virulence genes. Therefore, monitoring antimicrobial resistance in bird nests will provide important information on the distribution of antimicrobial resistance in the environment and the potential for spillover to humans. This surveillance strategy is particularly important in low and middle-income countries owing to the disproportionately higher burden of antimicrobial resistance and poor information on the environmental distribution of antimicrobial resistance (Iskandar et al., 2021; Sulis et al., 2022). Likewise, due to the less stringent environmental regulations and poor urban planning in low and middle-income countries, free-living birds are more likely to build their nest near human dwellings, significantly raising the potential for human exposure. This study evaluated the antibiotic profile of *E. coli* strains isolated from bird nests collected from the Akoko southwest area of Ondo State, Nigeria. The study aims to provide important information on the distribution of antibiotic resistance genes in the studied area and implications for public health by evaluating the antibiotic resistance profiles of *e. coli* strains isolated from bird nests

**2.0 Materials and Methods**

**2.1 Sample collections:** Bird nest samples were collected from locations in Akungba, Supare and Iwaro-oka Akoko in the Akoko Southwest area of Ondo State, Nigeria. Bird nests near human dwellings in the selected locations were collected between May and August 2022. The nests were collected using sterile gloves, and when not reachable by hand, the nests were collected using sterile containers attached to the end of an extendable pole. Bird nests were transported individually in sterile, labelled containers on ice packs to the laboratory for *E. coli* isolation. Samples were cultured for *E. coli* isolation within four hours of collection.

**2.2 Isolation of *E. coli* from bird nests:** Five gramsof the bird nests were suspended in 100 ml of sterile distilled water in sterile conical flasks. The flasks were wrist-shaken vigorously three times for 1 minute at 5-minute intervals to allow the suspension of the bird nest components. The suspensions were subsequently serially diluted in sterile distilled water. The samples were screened for *E. coli* using the pour plate technique. Briefly, 1 ml of the undiluted suspension and selected diluents (3 and 5) were aseptically transferred into sterile petri dishes in triplicate. Twenty ml of freshly prepared Eosin Methylene Blue (EMB) agar, which has been allowed to cool, was added to the plates and swirled gently to allow even spread of the media. The agar was allowed to solidify before the plates were inverted and incubated for 24 hours at 37°C. After incubation, the plates were examined for colonies showing the characteristic green metallic sheen of *E. coli* on EMB agar. The colonies were counted, and representative colonies were sub-cultured by streaking on freshly prepared EMB agar. The identity of the isolates was further confirmed based on their Gram staining reaction, cellular morphology and biochemical characteristics. The isolates were subjected to catalase, oxidase, indole, citrate, and urease tests.

**2.3 Antibiotic susceptibility test:** The susceptibility of the *E. coli* isolates to selected antibiotics was estimated using the disc diffusion assay according to (CLSI 2022). The inoculum used for the antibiotic susceptibility testing was prepared by inoculating colonies from freshly prepared plates into sterile Luria-Bertani (LB) broth. The inoculated broths were incubated overnight at 37°C, after which the optical density was adjusted to achieve a CFU/ml equivalent of the 0.5 McFarland standard. The standardised broths were inoculated onto freshly prepared Mueller–Hinton Agar (LAB M, UK). Sterile swab sticks were dipped into the standardised broth and thereafter pressed firmly against the sides to remove excess broth. The swab sticks were then used to swab the entire surface of the freshly Mueller–Hinton agar. The plates were allowed to dry for 30 minutes before antibiotic discs (Abteck, UK) were placed on the inoculated Mueller–Hinton agar and pressed firmly on the agar with sterile forceps to ensure complete contact with the agar. The plates were inverted and incubated overnight at 37°C for 24 hours, after which the plates were examined for zones of growth inhibition around the discs. The diameter of any zone of inhibition was measured using a calibrated ruler from the underside of the plate. The susceptibility of the isolates to the antibiotics was interpreted based on CLSI guidelines (CLSI, 2022).

**3.0 Result and Discussion**

This study examined the antibiotic susceptibility profile of *E. coli* isolated from bird nests in specific locations within the study area. A total of thirty-two bird nest samples were collected for analysis, as shown in Table 1. *E. coli* was isolated from 17 of these nests, resulting in a prevalence of 53.13%. The concentration of *E. coli* in the nests ranged from 2.0×10¹ to 2.8×10² CFU/g. Strains of *E. coli* have previously been isolated from free-living birds across diverse ecosystems and geographic regions (Rybak et al., 2022; Kobuszewska et al., 2024). This research provides valuable information on the prevalence of *E. coli* within the nests of free-living birds in the study area. The recovery of viable *E. coli* from nests indicates the potential for free-living birds to spread *E. coli* in environmental environments, as well as the capability of *E. coli* strains to survive under the environmental conditions present in the region. The microenvironment within bird nests is known to facilitate the persistence of microorganisms (Costanzo et al., 2022). The *E. coli* strains isolated may originate from bird droppings, other sources, or nesting materials such as dried grass, twigs, and mud.

The susceptibility of these *E. coli* strains to selected antibiotics is outlined in Table 2. Phenotypic resistance was observed in some isolates against all antibiotics except for imipenem. All isolates were susceptible to imipenem, whereas only 41.18% and 47.06% showed susceptibility to tetracycline and amoxicillin-clavulanate, respectively. Previously, *E. coli* strains resistant to various antibiotics have been isolated from wild birds across different regions of the world (Nowaczek et al., 2021; Rybak et al., 2022). Nowaczek et al. (2021) reported that 50% of *E. coli* strains from wild birds were resistant to tetracycline, with 46.8% resistant to ciprofloxacin. These findings suggest that wild birds, including those nesting near human habitats, are important reservoirs of resistance genes, likely acquired from environmental sources. Birds may forage in hotspots rich in antibiotic-resistant genes, such as landfills, polluted water bodies, or wastewater, ingesting resistant bacteria that colonise their gut and are subsequently excreted in their nests, creating a cycle of contamination. Resistance genes have also been detected in *E. coli* strains from wild birds and their habitats previously (Skarżyńska et al., 2021; Kumari et al., 2024).

The presence of resistant *E. coli* strains in bird nests highlights the potential for wild birds to spread antimicrobial-resistant microorganisms into the environment, which may spill over to humans either directly or indirectly. Bird nests, particularly those close to human settlements, may serve as microenvironments for the long-term survival of resistant bacteria, from where they can spread and infect humans (Mourkas et al., 2024). The antibiotic susceptibility profiles of *E. coli* from nests in this region also underscore potential ecological risks. Consequently, understanding the mechanisms of resistance and the factors driving the emergence of resistance in environmental niches is essential for developing effective management strategies.

**4.0 Conclusion**

The isolation of antibiotic-resistant *E. coli* strains from bird nests in this study showed that free-living birds nesting close to human dwellings may pose a risk of exposure to antibiotic-resistant bacterial strains to humans. Birds can transmit pathogenic bacteria to humans either through direct interaction or indirectly through various routes. Thus, it is crucial to monitor this potential spillover of pathogens from free-living birds to humans to assess its potential danger. Owing to the interconnectedness of human, animal, and environmental health, the results from this study underscore the need for a holistic approach to the control of antibiotic resistance through organised surveillance programs to monitor antibiotic resistance patterns in wildlife populations, particularly in areas where human-wildlife interactions are prevalent, such as the studied location.

**Table 1: Isolation of *Escherichia coli* from the bird nest samples**

|  |  |  |
| --- | --- | --- |
|  Samples | *E. coli* isolation | CFU/g |
| 1 | - | - |
| 2 | - | - |
| 3 | + | 6.0x101 |
| 4 | - | - |
| 5 | + | 4.0x101 |
| 6 | - | - |
| 7 | - | - |
| 8 | + | 2.8x102 |
| 9 | - | - |
| 10 | + | 4.0x101 |
| 11 | + | 2.0x102 |
| 12 | - | - |
| 13 | + | 8.0x101 |
| 14 | + | 1.2x102 |
| 15 | - | - |
| 16 | + | 2.0x101 |
| 17 | + | 4.0x101 |
| 18 | + | 6.0x101 |
| 19 | - | - |
| 20 | - | - |
| 21 | + | 1.6x102 |
| 22 | - | - |
| 23 | + | 2.0x101 |
| 24 | + | 1.2x102 |
| 25 | - | - |
| 26 | + | 4.0x101 |
| 27 | + | 8.0x101 |
| 28 | + | 2.0X101 |
| 29 | + | 1.2x102 |
| 30 | - | - |
| 31 | - | - |
| 32 | + | 6.0x101 |

**Table 2: Antibiotic Susceptibility Profile of *Escherichia coli* isolated from bird nests**

|  |  |  |  |
| --- | --- | --- | --- |
| Antibiotics  | Susceptible (%) | Intermediate (%) | Resistant (%) |
| Amoxicillin-clavulanate (20/10µg) | 47.06 (8) | 23.53 (4) | 29.41 (5) |
| Cefotaxime (30µg) | 66.67 (12) | 16.67 (3) | 16.67 (3) |
| Tobramycin (10 µg) | 83.33 (15) | 0.00 (0) | 16.67 (3) |
| Cefepime (30µg) | 64.71 (11) | 11.76 (2) | 23.53 (4) |
| Imipenem (10 µg) | 100.00 (18) | 0.00 (0) | 0.00 (0) |
| Norfloxacin (10 µg) | 50.00 (9) | 0.00 (0) | 50.00 (9) |
| Tetracycline (30µg) | 41.18 (7) | 35.29 (6) | 23.53 (4) |

**Disclaimer (Artificial intelligence)**

Author(s) hereby declare 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.

**References**

Anjum, M.F., Schmitt, H., Börjesson, S., Berendonk, T.U., Donner, E., Stehling, E.G., Boerlin, P., Topp, E., Jardine, C., Li, X. and Li, B., 2021. The potential of using E. coli as an indicator for the surveillance of antimicrobial resistance (AMR) in the environment. *Current Opinion in Microbiology*, *64*, pp.152-158.

Batista, R., Saraiva, M., Lopes, T., Silveira, L., Coelho, A., Furtado, R., Castro, R., Correia, C.B., Rodrigues, D., Henriques, P. and Lóio, S., 2022. Genotypic and phenotypic characterization of pathogenic Escherichia coli, Salmonella spp., and Campylobacter spp., in free-living birds in Mainland Portugal. *International Journal of Environmental Research and Public Health*, *20*(1), p.223.

Clinical and Laboratory Standards Institute. (2022). Performance Standards for Antimicrobial Susceptibility Testing. 32nd ed. CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA.

Costanzo, A., Ambrosini, R., Franzetti, A., Romano, A., Cecere, J.G., Morganti, M., Rubolini, D. and Gandolfi, I., 2022. The cloacal microbiome of a cavity-nesting raptor, the lesser kestrel (Falco naumanni). *PeerJ*, *10*, p.e13927.

Da Costa, P.M., Loureiro, L. and Matos, A.J., 2013. Transfer of multidrug-resistant bacteria between intermingled ecological niches: the interface between humans, animals and the environment. *International journal of environmental research and public health*, *10*(1), pp.278-294.

Fletcher, S., 2015. Understanding the contribution of environmental factors in the spread of antimicrobial resistance. *Environmental health and preventive medicine*, *20*, pp.243-252.

Iskandar, K., Molinier, L., Hallit, S., Sartelli, M., Hardcastle, T.C., Haque, M., Lugova, H., Dhingra, S., Sharma, P., Islam, S. and Mohammed, I., 2021. Surveillance of antimicrobial resistance in low-and middle-income countries: a scattered picture. *Antimicrobial Resistance & Infection Control*, *10*, pp.1-19.

Kobuszewska, A. and Wysok, B., 2024. Pathogenic Bacteria in Free-Living Birds, and Its Public Health Significance. *Animals*, *14*(6), p.968.

Kumari, A., Singh, N.A. and Koli, V.K., 2024. Avian reservoirs or vectors? Unravelling the relationship between waterbirds and E. coli contamination. *Ornithology Research*, *32*(1), pp.1-10.

Mourkas, E., Valdebenito, J.O., Marsh, H., Hitchings, M.D., Cooper, K.K., Parker, C.T., Székely, T., Johansson, H., Ellström, P., Pascoe, B. and Waldenström, J., 2024. Proximity to humans is associated with antimicrobial-resistant enteric pathogens in wild bird microbiomes. *Current Biology*, *34*(17), pp.3955-3965.

Naghavi, M., Vollset, S.E., Ikuta, K.S., Swetschinski, L.R., Gray, A.P., Wool, E.E., Aguilar, G.R., Mestrovic, T., Smith, G., Han, C. and Hsu, R.L., 2024. Global burden of bacterial antimicrobial resistance 1990–2021: a systematic analysis with forecasts to 2050. *The Lancet*, *404*(10459), pp.1199-1226.

Nowaczek, A., Dec, M., Stępień-Pyśniak, D., Urban-Chmiel, R., Marek, A. and Różański, P., 2021. Antibiotic resistance and virulence profiles of Escherichia coli strains isolated from wild birds in Poland. *Pathogens*, *10*(8), p.1059.

Nyirabahizi, E., Tyson, G.H., Dessai, U., Zhao, S., Kabera, C., Crarey, E., Womack, N., Crews, M.K., Strain, E. and Tate, H., 2020. Evaluation of *Escherichia coli* as an indicator for antimicrobial resistance in Salmonella recovered from the same food or animal ceca samples. *Food Control*, *115*, p.107280.

Ramos, S., Silva, V., Dapkevicius, M.D.L.E., Caniça, M., Tejedor-Junco, M.T., Igrejas, G. and Poeta, P., 2020. *Escherichia coli* as commensal and pathogenic bacteria among food-producing animals: Health implications of extended spectrum β-lactamase (ESBL) production. *Animals*, *10*(12), p.2239.

Rybak, B., Krawczyk, B., Furmanek-Blaszk, B., Wysocka, M., Fordon, M., Ziolkowski, P., Meissner, W., Stepniewska, K. and Sikorska, K., 2022. Antibiotic resistance, virulence, and phylogenetic analysis of Escherichia coli strains isolated from free-living birds in human habitats. *PLoS One*, *17*(1), p.e0262236.

Skarżyńska, M., Zaja̧c, M., Bomba, A., Bocian, Ł., Kozdruń, W., Polak, M., Wia̧cek, J. and Wasyl, D., 2021. Antimicrobial resistance glides in the sky—free-living birds as a reservoir of resistant Escherichia coli with zoonotic potential. *Frontiers in Microbiology*, *12*, p.656223.

Smith, O.M., Snyder, W.E. and Owen, J.P., 2020. Are we overestimating risk of enteric pathogen spillover from wild birds to humans?. *Biological Reviews*, *95*(3), pp.652-679.

Sulis, G., Sayood, S. and Gandra, S., 2022. Antimicrobial resistance in low-and middle-income countries: current status and future directions. *Expert review of anti-infective therapy*, *20*(2), pp.147-160.

Xin, J., Cao, H., Bao, X. and Hu, C., 2023. Does nest occupancy by birds influence the microbial composition?. *Frontiers in Microbiology*, *14*, p.1232208.

Zabłotni, A., Kaliński, A., Bańbura, M., Glądalski, M., Markowski, M., Skwarska, J., Wawrzyniak, J. and Bańbura, J., 2020. Experimental nest replacement suggests that the bacterial load of nests may mediate nestling physiological condition in cavity nesting Great Tits (Parus major). *Journal of Ornithology*, *161*, pp.819-828.