

Dear Reviewer,

Thank you for your comments and suggestions. We have carefully considered each point and made the necessary revisions to the manuscript (in the manuscript please see the text formatted in purple, as given by track changes). Our responses to your observations are provided below, in blue.

A One Health comparative study of MDR *Escherichia coli* 2 isolated from clinical patients and farm animals in Satu Mare, 3 Romania

The study aimed to compare antimicrobial resistance (AMR) profiles of *E. coli* isolates from hospitalized patients and food-producing animals in Satu Mare, a county located in north-western Romania. The study underscores the veterinary sector as a key contributor to the maintenance and spread of MDR *E. coli*, and the need for stricter antimicrobial use policies in livestock to reduce transmission risks across human and animal populations.

Abstract:

- Could you please specify the source of the *coli* isolates recovered in hospitals (e.g., swab samples, stool, instruments, etc.)? I would also appreciate the same information regarding the animal isolates.

We thank for the reviewer for the observation. Please see the abstract with the details mentioned added (lines 20-24).

- Regarding statistical analysis, significant and non-significant differences ($P < 0.05$) should be interpreted and reported in the abstract when comparing outcome results

We appreciate the reviewer's observation. Please see again the Abstract with the statistical analysis details added (line 32).

Introduction:

- Could you please clarify the definition of the term 'One Health challenge' and provide the source of this terminology?

We thank the reviewer for this question. The article was initially included in the special issue of Antibiotics journal, called "Veterinary Microbiology and Antimicrobial Resistance – the One Health Approach" but after for unknown reason was taken out and included in no special issue. Thus, this was one reason the term of One health was approached and included also in the title and, on the other hand, because nowadays when the subject of antibiotic resistance is approached is correlated with One Health challenge

to stop the spread of antibiotic resistance across the 3 sectors included in this concept (humans, animals and environment).

The term *One Health challenge* refers to a health or environmental problem that arises at the interface between human, animal, plant and ecosystem health — for example zoonotic disease emergence, antimicrobial resistance, food and water security, or climate-driven vector spread — and which can only be effectively addressed by applying a One Health approach: a multisectoral, interdisciplinary, collaborative method as defined by the WHO and the One Health High-Level Expert Panel (OHHLEP).

https://www.who.int/news/item/01-12-2021-tripartite-and-unep-support-ohhlepe-s-definition-of-one-health?utm_source=chatgpt.com

- **Objectives:** Could you please clarify why the investigators chose to study only one bacterial strain (*E. coli*)? What was the rationale for excluding other Gram-negative bacteria such as *Salmonella* or *Campylobacter*, as well as Gram-positive bacteria like *Staphylococcus* or *Clostridium*? It would have been preferable for the investigators to include at least one additional Gram-negative and/or Gram-positive bacterial strain to strengthen and support their findings.

We thank the reviewer for pointing this out and the opportunity to clarify our study design. The present work focused exclusively on *Escherichia coli* because it represents one of the most important indicator organisms for monitoring antimicrobial resistance within the One Health framework. *E. coli* is widely recognized as a sentinel bacterium for both commensal and pathogenic populations, being commonly found in humans, animals, and the environment. Its well-characterized resistance mechanisms make it an ideal model for comparative studies investigating the possibility of dissemination of multidrug resistance across sectors. This study is part of a larger research project that specifically targets *E. coli* strains, including both commensal and β -lactamase-producing isolates, collected from clinical patients and farm animals in Satu Mare County, Romania. The inclusion of additional Gram-negative (e.g., *Salmonella*, *Campylobacter*) or Gram-positive bacteria (e.g., *Staphylococcus*, *Clostridium*) was beyond the scope of the current study but are already considered for future work to provide broader comparative insights across bacterial species.

- **Line 89:** In addition to reducing antibiotic use, there are other strategies that can help control the transfer of resistance genes. Could you please specify which approaches you consider essential for limiting the spread of antimicrobial resistance, and explain how these align with your core objectives and findings?

We appreciate the reviewer's observation. Details about the strategies that can help control the transfer of resistance genes and the essential approaches for limiting the spread of antimicrobial resistance in concordance with our study were added. Please see in the Introduction section the lines 108-122.

- **Line 95:** what about cattle farms?

We thank the reviewer for the question. The cattle weren't mentioned because there weren't any samples from the cattle within this research, but please see the Introduction section lines 125-127 with reference for cattle farms added.

- **Line 101:** Therefore, this study "could represent" one of the first systematic...

We thank the reviewer for the brief observation. Please see the line 131 corrected.

- **Objectives:** A major concern is that the investigators studied antimicrobial resistance patterns only phenotypically. It is essential to perform genetic identification of resistance mechanisms and correlate those findings with resistance patterns in both human and animal isolates. It was essential to identify the antimicrobial resistance genes and correlate them with the phenotypic results for both human and animal isolates.

We appreciate the reviewer for the valuable observation. We agree that molecular characterization of antimicrobial resistance determinants would have provided deeper insights into the underlying genetic mechanisms and potential transmission pathways between human and animal isolates. As noted in the limitations section of our manuscript (lines 572-582), and also in the objectives of the study (line 137) our research focused on phenotypic resistance profiling due to resource and infrastructure constraints. Nevertheless, we fully acknowledge that future work incorporating molecular analyses (such as detection of resistance genes and genotyping or whole-genome sequencing) would be crucial to corroborate the phenotypic findings, identify specific resistance determinants, and better understand possible clonal relationships or cross-species transmission dynamics.

Methodology:

- **Table 5:** Could you please specify the number and percentage of clinical samples according to specimen type?

We thank the reviewer for this suggestion. Unfortunately, we do not have detailed information on the specimen type for each clinical sample, as the samples were reported to us by the laboratory within the hospital, as hospital department rather than by specimen type. Table 5 presents the number and origin of human samples according to

hospital department, reflecting the data provided by the reporting laboratory. We have clarified this in the manuscript to ensure transparency (lines 610-611).

- Line 419 mentions 550 human samples, whereas Table 5 indicates 571 human samples. Could you please clarify this discrepancy.

We thank the reviewer for the observation. Please see the correction of table 5.

- Could you please specify the source of the *coli* isolates (specimen type) recovered from animals?

As it mentioned in in subchapter 4.3 Animal sampling and isolate collection, the specimen types were the ceca harvested from healthy animals, examined by licensed veterinarians who performed ante-mortem inspections. Thus, the specimen type was the cecal contents (feces).

- Could you please clarify why the number of animal samples differed significantly?

The variation in sample numbers reflects the sampling framework established under the DECISION (EU) 2020/1729 of 17 November 2020 on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria. According to this decision, *Escherichia coli* isolates are to be collected from animals slaughtered in slaughterhouses—specifically poultry, pigs, and cattle less than one year old. Our study followed these official guidelines and was conducted over a two-year period. The number of samples obtained from each animal category was therefore proportional to the number of animals slaughtered during that timeframe in the Satu Mare region. Consequently, the differences in sample numbers among species reflect the actual slaughter patterns and population distribution rather than a methodological imbalance.

- Could you please provide the reference for the isolation and identification of *coli*? Additionally, was it important to include advanced verification procedures (PCR) to identify *E. coli* and establish genetic correlations between different strains?

We thank the reviewer for the question. Please see the reference regarding the isolation and identification of *E. coli*. In the present study, the identification of *E. coli* isolates was performed using conventional biochemical methods, as described in the Materials and Methods section. These procedures are standard and widely accepted for the identification of *E. coli* within antimicrobial resistance monitoring programs and provide reliable species-level confirmation. Although advanced molecular techniques (such as PCR) can offer additional verification and allow for genetic correlation analyses, this was a limitation of this study. The main objective was to perform a phenotypic characterization and comparative assessment of multidrug-resistant *E. coli* isolates from human and animal sources. Future studies within the larger research framework will

include molecular analyses to further explore genetic relationships and resistance mechanisms.

https://www.food.dtu.dk/english/-/media/institutter/foedevareinstituttet/temaer/antibiotikaresistens/eurl-ar/protocols/esbl-ampc-and-camrbapenemase-producing-e-coli/esbl_ampc_cpeprotocol_version_caecal_v9_17122024.pdf

- Antimicrobial susceptibility testing: Could you please clarify why the authors used minimum inhibitory concentrations (MICs) for animal isolates? Did they assess antimicrobial resistance using CLSI breakpoints for animal isolates as they did for human isolates?

We thank the reviewer for this comment and the opportunity to clarify our methodological approach. The animal isolates were processed in a veterinary diagnostic laboratory that routinely determines antimicrobial susceptibility using the minimum inhibitory concentration (MIC) method. This method is performed according to protocol no. 9 of 2024, which specifies the use of the broth microdilution plate method for isolates obtained from animals within the European Union. This protocol follows the recommendations of the European Union Reference Laboratory for Antimicrobial Resistance (EURL-AMR) and represents the standard procedure for antimicrobial susceptibility testing in animal isolates across EU member states. Furthermore, the interpretation of antimicrobial resistance results for animal isolates was carried out according to both CLSI and EUCAST guidelines, ensuring methodological consistency and comparability with the human isolates analyzed in this study.

- Line 453: Could you please clarify the reason for choosing these antibiotics to assess antimicrobial resistance?

We thank the reviewer for this pertinent question. The selection of antimicrobial substances in our study was based on the requirements of the DECISION (EU) 2020/1729 of 17 November 2020, which establishes the framework for the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria isolated from animals. This decision specifies the antimicrobial classes to be included in national and EU-level AMR surveillance programs, ensuring comparability of data across countries and sectors. In addition, to enable a meaningful comparative analysis between animal and human isolates, we selected the same categories of antimicrobials for both populations. This approach allowed us to directly assess similarities and differences in resistance patterns across the human–animal interface within the One Health context. Please see details added at Antimicrobial susceptibility testing (AST) section (Materials and methods) lines 652-655.

- What was the source of the antibiotic discs?

Please see the source of antibiotics discs, which was added at line 651 in the materials and methods section.

<https://www.bio-rad.com/en-ro/product/antimicrobial-susceptibility-disks?ID=LWH2W7ESH>

- What was the concentration of each antibiotic used?

Please see the concentrations which are found in Table no. 2 of the DECISION (EU) 2020/1729.

<https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32020D1729>

- Resistance category definitions: Could you please provide the definition of MDR according to CLSI (2025)?

For the purposes of this study, multidrug-resistant (MDR) isolates are defined as those exhibiting acquired non-susceptibility to at least one agent in three or more antimicrobial categories, according to widely-accepted international definitions. While CLSI's M100 (35th Edition, 2025) provides authoritative breakpoints for individual antimicrobials, it does not currently include a comprehensive, standardized MDR-category definition applicable across all bacterial taxa. Therefore, for consistency and comparability, the classification of MDR in this study followed the criteria established by Magiorakos et al. (2012, Clin Microbiol Infect 18:268–281), which is widely used in both human and veterinary antimicrobial resistance research.

- How many independent experimental trials were conducted to perform this study?

We thank the reviewer for this question. Each isolate was tested once for antimicrobial susceptibility as part of the standard laboratory workflow. However, samples showing unusual or inconsistent resistance patterns, more precisely the susceptibility to ampicillin, but resistance to certain cephalosporins, or variable resistance among structurally similar cephalosporins were retested in duplicate to confirm the accuracy of the results. Therefore, while most tests were performed once, a subset of isolates was analyzed twice independently to ensure the reliability and reproducibility of the data.

Results and discussion

- Line 112: Please note that the 571 samples reported in Table 5 were collected from human specimens and are not related to the animal samples mentioned in lines 112-113. Table 5 specifically reports samples collected from human specimens, totaling 571 samples. Is that correct? Please clarify and re-check the sample numbers throughout the manuscript.

We thank the reviewer for this observation. Corrections were made in table 5, as the correct number of human samples was 550 (please see again the table 5), while the total isolates of *E. coli* was 571 (420 from human and 151 from animal).

- Table 3: Please clarify what the numbers presented represent, as it is not clear. Also, please provide the reference for the resistance profile? I would also appreciate the same regarding Table 4.

We thank the reviewer this helpful observation. The numbers in Table 3 represent the number of *E. coli* isolates found to be resistant to each antimicrobial agent (AMK: amikacin; AMP: ampicillin; CTX: cefotaxime; CAZ: ceftazidime; FEP: cefepime; CIP: ciprofloxacin; GEN: gentamicin; SXT: trimethoprim–sulfamethoxazole) in each hospital department. The final row indicates the overall prevalence (%) of resistance for each antimicrobial among all *E. coli* isolates tested. The interpretation of antimicrobial susceptibility results and classification of resistance were performed according to the CLSI M100, ed. 35 (2025) and/or EUCAST v15.0 guidelines (mentioned also in material and methods section) (reference added in the revised manuscript). The same clarification and reference have been added to Table 4. Please see again table 3 and 4.

- The Results section should focus solely on presenting the major findings; however, the authors have included extensive discussion in this section, which should be moved to the Discussion section!

We thank the reviewer for this comment. Upon careful review, we found that the Results section primarily presents the major findings, with minimal discussion included. To further address the concern, we have expanded and clarified the Discussion section, providing additional interpretation and context where appropriate. From our point of view, no content was needed to be moved from the Results, as it already focused on presenting the data.

- The authors should clearly explain why animal isolates consistently display higher resistance levels than human isolates and correlate their findings with existing published data?

We appreciate the reviewer's observation. The entire Discussion section was revised and a dedicated paragraph has been added to this section in providing a detailed explanation and interpretation of the higher resistance levels in animal isolates. This interpretation is directly correlated with published data on antibiotic usage and surveillance in the veterinary sector. Please see again the Discussion section.

- A more thorough discussion and interpretation of the results should be provided in the Discussion section, with appropriate use of statistical analysis and data interpretation! The

current discussion reads more like a literature review than a focused interpretation of the study's findings!

We acknowledge this critical and highly valuable feedback. The Discussion section has been completely restructured and rewritten to address this concern directly. We have prioritized the focused interpretation of our study's findings over a general literature review. The revised content, now clearly integrates the results of our statistical analysis and provides a detailed interpretation of the key data trends, particularly concerning the observed resistance patterns. We believe the revised Discussion now serves as a focused analysis and comparison of our results, as requested.

- As mentioned earlier, a major concern is that the investigators did not perform gene analysis to study antimicrobial resistance genes, correlate them with phenotypic results, or explore genetic relationships between different human and animal isolates.

We appreciate the reviewer's insightful comment. We recognize that genetic analysis of antimicrobial resistance determinants and their correlation with phenotypic profiles, as well as exploration of genetic relatedness among isolates, would have added substantial value to our findings. However, the primary objective of the present study was to establish baseline data on phenotypic antimicrobial resistance patterns among human and food-producing animal isolates in the study area, which had not been previously documented. Given the limited funding available at the time, molecular investigations were beyond the scope of this work. We have already acknowledged this limitation in the revised manuscript (lines 574-577). We agree that integrating molecular characterization—such as resistance gene detection, genotyping, or whole-genome sequencing—should be a priority in future research to provide a more comprehensive understanding of resistance mechanisms and potential transmission dynamics (lines 580-582).

Conclusion:

- It is clear that 30% of human isolates were non-MDR, given that 70% were MDR. Please consider deleting the phrase '30% were non-MDR.'

The recommendation of the reviewer was applied and deletion of 30% were non-MDR was done. Please see again the Conclusions section.

- Since 79.74% of the animal strains were MDR, approximately 20% were considered non-MDR. Could you please clarify the status of the 9.68% identified as non-MDR isolates?

We appreciate the valuable comment. The conclusion was corrected, as the MDR isolates from animals had a percentage 79.47%, the non-MDR had a percentage of 11.25% and 9.27% were fully susceptible.

- In general, the conclusion should be concise and directly related to the study findings. Could you please rewrite the conclusion to be brief, precise, and free of repetition?

We appreciate the recommendation, the conclusions were adapted and we think free of repetition now. Please see again, the Conclusions section.