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Article

Investigation of microbial resistance and the "microbial footprint" in intensive livestock farming units in West Greece

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Abstract
The extensive use of antibiotics in intensive livestock production has contributed significantly to the emergence and spread of antimicrobial resistance, which constitutes a major global public health concern. Indicator bacteria such as Escherichia coli and Enterococcus spp. are commonly used to assess fecal contamination and to reflect the selective pressure exerted by antimicrobial agents on the gastrointestinal microbiota of food-producing animals. The aim of this study was to map the "microbial footprint" and evaluate antibiotic selective pressure in pig and laying hen farms in the Arta region of Western Greece through a comparative analysis of resistance patterns in E. coli and Enterococcus isolates. The results revealed widespread antimicrobial resistance across all samples. E. coli isolates from pig farms exhibited particularly high levels of multidrug resistance, with MAR index values ranging from 0.53 to 0.62, indicating intense and sustained antibiotic pressure. Enterococcus spp. displayed lower but consistently elevated resistance, especially to sulfonamides. Overall, the findings demonstrate that antimicrobial resistance is a stable characteristic shaped by bacterial species and farm management practices, underscoring the need for systematic surveillance and rational antibiotic use in livestock production systems.

Keywords: *antimicrobial resistance, Escherichia coli, Enterococcus spp., MAR index, livestock production*

INTRODUCTION

The initial use of antibiotics in diets arose from the discovery in the late 1940's, in the United States, that including the fermentation products of Streptomyces aureofaciens (a strain of bacteria) in the diets of simple-stomached animals, such as pigs and poultry, resulted in growth responses. This practice quickly became widespread due to its significant benefits in productivity, which significantly impacted intensive farming systems [1÷3]. In the next fifty years, the use of antibiotics as feed additives in livestock production became virtually universal. However, antibiotic resistance increased worldwide at an accelerating pace, reducing the efficacy of therapies for many infections, fueling the transmission of epidemic pathogens, and increasing healthcare costs, morbidity, and mortality related to infectious diseases [4÷6]. In the 1980s, the emergence of antimicrobial resistance caused a major shift in the search for new antimicrobial agents, leading to the development of cephalosporins and fluoroquinolones. These classes of antibiotics were designed to combat resistant pathogens and have since become an integral part of the treatment of various infections [7÷10]. Public health consequences arising from the excessive use of antimicrobials in livestock production include the emergence of resistant bacteria, which can subsequently be transferred to humans through the food chain [11,12].

Resistant strains of four bacteria that cause disease in humans have been transmitted from animals, leading to significant consequences for public health. These include Salmonella, Campylobacter, Enterococcus, and E.coli [13÷16]. The World Health Organization (WHO) has concluded that antimicrobial resistance is a serious and complex global problem, requiring the establishment of a global surveillance system in both veterinary and human medicine. This public health threat has been

recognized as a priority for intervention by health agencies at national and international levels [17]. E.coli and Enterococcus spp. are found in the gastrointestinal tracts and feces of all warm-blooded animals and some cold-blooded animals [18÷21]. These bacteria are used as indicators of fecal contamination in polluted waters [22].

Resistance to multiple types of antibiotics is not uncommon in E. coli and Enterococcus strains isolated from animals and humans. The selective pressure imposed on the commensal gastrointestinal flora of animals and humans by antibiotic use results in patterns of antibiotic resistance that reflect to some extent the microflora's exposure to antibiotics [23]. There are four general mechanisms of resistance, all of which are controlled by the action of specific genes: enzymatic inactivation or modification of antimicrobial agents, impermeability of the bacterial cell wall or membrane, active expulsion of the drug by the cellular efflux pumps, and alteration of target receptors [24÷26]. The transfer of resistance genes as well as the already resistant bacteria themselves is particularly favoured by the presence of antibiotics over a long period and at subtherapeutic concentrations. These factors play a role in the stimulation of resistance and its transfer through genetic material in bacteria. Exposure of bacteria to such subtherapeutic antimicrobial concentrations is thought to increase the speed with which resistant bacterial strains are selected, for example, if antibiotics are used as growth promoters or by improper use in veterinary medicine and human medicine [27].

There are few reports on the resistance of bacteria to antibiotics in Greece, and most of them focus on antimicrobial resistance in humans (patients, children) and drinking water samples, rather than on agricultural animals [28÷31]. According to these reports, the most widely used methods are the Kirby–Bauer method (a qualitative plate disc diffusion method) and microdilution susceptibility tests, which determine the minimum inhibitory concentration against the organism being tested.

This article differs from others in Greece because it analyzes antibiotic resistance patterns of E.coli and Enterococcus from two of the most common types of agricultural animals (pigs and laying hens) in Western Greece, using a larger panel of fifteen antibiotics, each tested at four concentrations, and compares the multiple antibiotic resistance profiles of these gastrointestinal bacteria. This study was based on the method of antibiotic resistance analysis described by Wiggins and Harwood [32,33]. The comparison of multiple antibiotic resistance profiles of E. coli and Enterococcus can be an important and useful tool to determine the frequency of antibiotic use in food-producing animals and, furthermore, to assess whether there is an inclination for the transfer of resistance between Gram groups of bacteria.

MATERIALS AND METHODS

Sample collection

Fecal samples were collected from six distinct geographical locations within the Arta region. Fresh feces were obtained from two livestock categories: pigs and laying hens. Specifically, one sample was collected from each of the six different farms per animal category (three pig livestock farms and three livestock laying hen farms). After collection, all samples were placed on ice in a cooler, transported to the laboratory for analysis and processed within 6 hours.

Isolation of E. coli and Enterococcus

For all farms, fecal samples were processed using the same standardized isolation protocol. Depending on sample consistency, an amount of fecal material (0.1 to 1.0 g) for E. coli isolation was suspended in 1 litter of buffered water (0.0425 g/L KH_2PO_4 and 0.4055 g/L $MgCl_2$) and filtered through 0.45-μm-pore-size filters (type GN-6; Pall Life Sciences). The filters were then transferred to 50-mm petri dishes (Bioster S.p.A., Italy, BG) containing Chromocult® Coliform Agar (Merck KGaA/MilliporeSigma, Darmstadt, Germany) and were then placed in the incubator for 24 hours at 44.5ºC, a selective temperature commonly used for the isolation of thermotolerant E. coli and fecal coliform bacteria [18,19]. For Enterococcus isolation the fecal samples were suspended in 1 liter of saline buffer (8.5 g/L NaCl, 0.3 g/L KH_2PO_4, and 0.6 g/L Na_2HPO_4 [pH 7.3]) and filtered. The filters were transferred to petri dishes containing Enterococcus Confirmatory Agar (Difco Laboratory, Detroit, MI USA) and incubated for 48 hours at 37ºC. After incubation, 96 isolated E. coli colonies

(dark-blue to violet colonies) and 96 Enterococcus colonies (red-pigmented colonies) from each sample were picked at random with sterile toothpicks and transferred to microwell plates containing 0.2 mL broth (EC Broth for E. coli and Enterococcosel broth for Enterococcus), resulting in a total of 288 isolates per bacterial species for pig farms and 288 isolates per bacterial species for laying hen farms. The microwell plates were then incubated at 37°C for 24 hours.

Determination of antibiotic resistance pattern

The antibiotics were selected because of their widespread use in veterinary and human medicine. In addition, certain compounds such as cefadroxil (CED) and cefaclor (CEC) were included as representative first- and second-generation cephalosporins commonly used in human and companion animal medicine, in order to assess broader β-lactam resistance patterns and potential cross-resistance mechanisms among isolates. To determine the antibiotic resistance patterns, the bacterial isolates were inoculated onto 100-mm diameter Trypticase Soy Agar (TSA) plates amended with specific concentrations of antibiotics. This inoculation was performed using a sterile 48-prong replicator (Sigma Chemical Inc.), which allowed the simultaneous and precise transfer of multiple isolates onto each agar plate. Control TSA plates without antibiotics were also inoculated using the same replicator device to verify isolate viability. Specifically, the resistance profile of each isolate was evaluated against the following antibiotics: amoxicillin (AMX) at 20, 30, 50, and 80 μg/mL; ampicillin (AMP) at 10, 15, 30 and 50 μg/mL; gentamycin (GEN) at 10, 30, 60 and 80 μg/mL; erythromycin (ERY) and sulfachloropyridazine (SUC) at 10, 20, 30 and 50 μg/mL; cefadroxil (CED), cefaclor (CEC), and chloramphenicol (CPN), 10, 20, 40 and 60 μg/mL; neomycin (NEO) at 10, 30, 40 and 50 μg/mL; oxytetracycline (OTC), penicillin (PEN), sulfamethoxazole (SUM), streptomycin (STR), tetracycline (TET), and chlorotetracycline (CTC) at 20, 40, 60 and 80 μg/mL. Isolates were also spotted onto a control TSA plate containing no antibiotic. All plates were incubated for 24 hours at 37ºC. The colonies were recorded as positive if there was growth or negative if there was no growth. Isolates that did not grow on the control plates were excluded from the analysis.

Statistical analysis

To minimize the risk of pseudo-replication, antibiotic resistance data were summarized at the farm level. All statistical analyses were performed with farms treated as the independent experimental units. Data for antibiotic resistance pattern was analyzed using SPSS software (version 13.0; SPSS Institute Inc.). Pearson's rank correlation analysis was used to test the relationship between E. coli and Enterococcus strains percentage of antibiotic resistance isolates from all types of sources. Also, data of each of the source isolates were analyzed by using Cluster analysis. Cluster analysis is a multivariate technique that groups variables (isolates) with similar resistance profiles [34÷36]. This method constructs a database in which isolates are clustered according to their source, joining clusters in a manner that maximizes similarity within groups. The cluster analysis procedure generates a dendrogram that initially groups identical or highly similar isolates and subsequently organizes these groups into higher-level clusters.

Evaluation of antibiotic resistance patterns and public health risks using the Multiple Antibiotic Resistance (MAR) index

The present study utilized the Multiple Antibiotic Resistance (MAR) index, a critical tool for identifying high-risk sources of contamination by providing insights that conventional bacterial quantification methods may overlook. The MAR index underlines the necessity for stringent surveillance and management protocols, particularly in clinical settings where antibiotic pressure is high [37]. The MAR index was calculated for each bacterial isolate (E. coli and Enterococcus strains) and source using the following formula (1), according to [38]:

$$MAR = a / b \text{ X } c \quad (1)$$

where a represents the aggregate sum of resistance instances observed across all tested antibiotics, b is the total number of antibiotics to which the isolates were exposed and c is the number of isolates or concentration levels tested for each category.

The interpretation of MAR values is critical for risk assessment. Specifically, MAR ≤ 0.200 indicate low-risk sources with rare or no antibiotic use, whereas MAR > 0.200 indicate high-risk sources associated with frequent antibiotic use, such as human sewage, poultry farms, and pig farms [39÷41].

RESULT AND DISCUSSION

Antimicrobial agent resistance was noticed in all collected sample types. Initially, the homogeneity of the samples in terms of antibiotic resistance was estimated for both *E. coli* and *Enterococcus* strains. Although fecal samples were collected from different geographical locations within the Arta region, the results of the Pearson correlation coefficient analysis indicated no significant difference in the samples of each agricultural animal group ($p < 0.01$) (Table 1a÷d). Correlation analysis of the samples indicated a high degree of homogeneity among them, regardless of geographical origin, suggesting that antibiotic resistance is a stable characteristic of microbial populations rather than a random or strictly local phenomenon. This finding supports the view that agricultural practices and the systematic use of antibiotics create a selective environment that favors resistant strains, thereby outweighing the influence of environmental factors.

Table 1. Pearson correlation analysis of antibiotic resistance patterns for *E.coli* and *Enterococcus* isolates from pigs (PG) and laying hens (HL)

Table 1a. *E.coli* isolates from pigs (Pigs – PGEC)

	PGECI	**PGECII**	**PGECIII**
PGECI	-		
PGECII	0.898**	-	
PGECIII	0.870**	0.967**	-

**Correlation is significant at the 0.01 level (2-tailed).

Table 1b. *Enterococcus* isolates from pigs (Pigs – PGE)

	PGEI	**PGEII**	**PGEIII**
PGEI	-		
PGEII	0.888**	-	
PGEIII	0.889**	0.946**	-

**Correlation is significant at the 0.01 level (2-tailed).

Table 1c. *E.coli* isolates from laying hens (Laying Hens – HLEC)

	HLECI	**HLECII**	**HLECIII**
HLECI	-		
HLECII	0.956**	-	
HLECIII	0.933**	0.912**	-

**Correlation is significant at the 0.01 level (2-tailed).

Table 1d. *Enterococcus* isolates from laying hens (Laying Hens – HLE)

	HLEI	**HLEII**	**HLEIII**
HLEI	-		
HLEII	0.954**	-	
HLEIII	0.915**	0.933**	-

**Correlation is significant at the 0.01 level (2-tailed).

All the types of fecal samples and animal species groups indicated that antibiotic resistance in *E. coli* isolates was more prevalent than in *Enterococcus* isolates as has been demonstrated in the international literature [42÷45]. The results of the present study on the resistance profile of bacteria isolated from pigs demonstrate a clear differentiation between the two species examined. Specifically,

E. coli strains displayed extensive resistance to a wide range of antimicrobial agents, with resistance rates approaching or exceeding 80÷95% for the antibiotics AMX, AMP, ERY, OTC, PEN, SUM, SUC, TET and CTC. Conversely, lower resistance levels were observed for GEN, CED, CEC and NEO. Regarding the *Enterococcus* isolates, they showed significantly more limited and selective resistance, with the highest values (60÷80%) being found for OTC, STR, TET, SUC and SUM, while for the other antibiotics the rates remained at low levels, often below 30% (Figure 1).

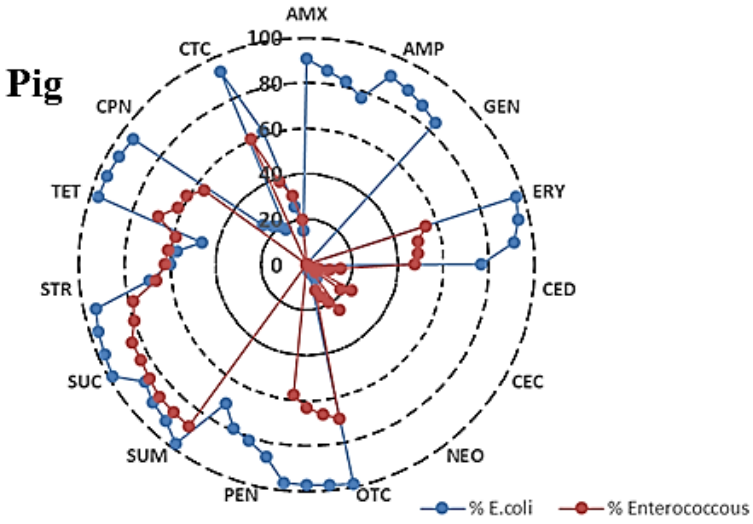

Fig. 1. Mean resistance rates (%) of *E. coli* and *Enterococcus* bacterial isolated from pigs' fecal samples against various antibiotics

In laying hens, however, the results are reversed. The resistance profiles of *E. coli* across the various antibiotics tested were generally low to moderate, with resistance rates ranging from 10% to 30%. However, exceptionally high resistance was recorded against ERY, reaching approximately 98%. Moderate resistance levels were also observed for antibiotics such as TET and STR, while low rates were noted for PEN and CPN. Regarding *Enterococcus*, the data show higher resistance rates compared to *E. coli* for certain antibiotics. Particularly high resistance was observed for STR, SUC, and SUM. Conversely, low resistance rates were recorded for several other antibiotics, including AMX, AMP, and GEN (Figure 2).

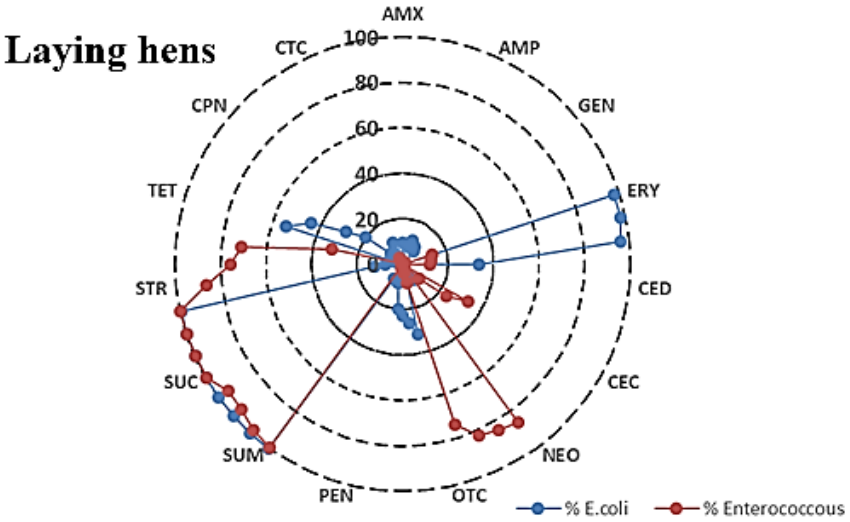

Fig. 2. Mean resistance rates (%) of *E. coli* and *Enterococcus* strains isolated from laying hens' fecal samples against various antibiotics

Analysis of the antibiotic resistance profile of *E. coli* strains revealed high levels of multidrug resistance, with a clear predominance of isolates from pigs compared to those from laying hens (Figure 3). In the pig population, *E. coli* exhibits an extremely high frequency of resistance (often exceeding 80÷90%) to a wide range of antimicrobial agents. In particular, resistance rates to penicillins (AMX, AMP, PEN) approach 90%, while a similar trend is observed for ERY. The highest antibiotic resistance levels in pigs are recorded for tetracyclines (OTC, TET, CTC) and sulfonamides (SUM, SUC), with rates approaching 100%.

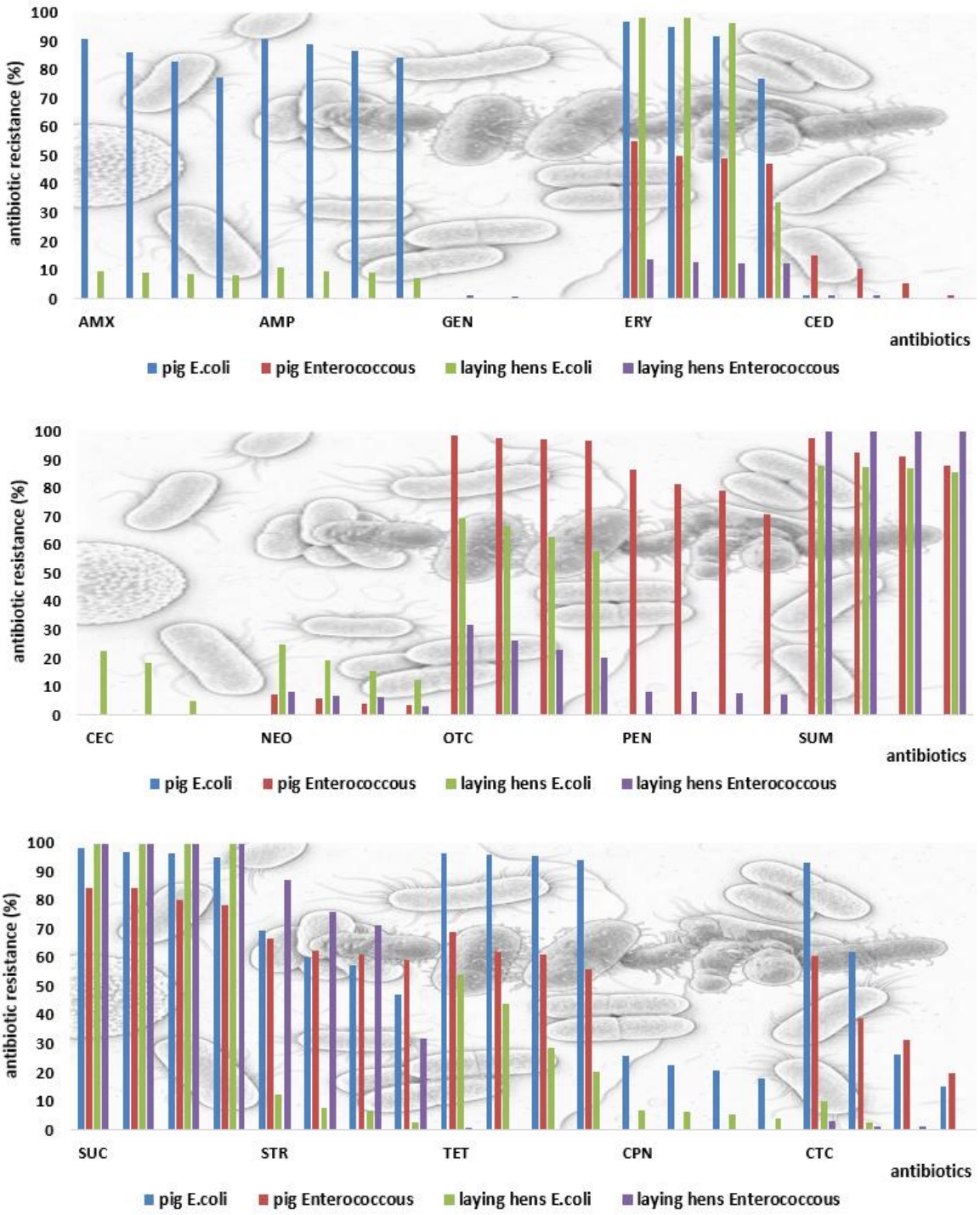

Fig. 3. Comparative percentage of mean antibiotic resistance (%) in *E. coli* and *Enterococcus* strains isolated from pigs and laying hens against selected antibiotics

In contrast, zero resistance was observed against GEN, CED, and CEC, while low resistance was noted for NEO and CPN. In laying hens, *E. coli* generally exhibits a more favorable resistance profile,

with the exception of two specific antimicrobial classes. These strains demonstrate almost universal resistance (~100%) to ERY and sulfonamides (SUM, SUC), reaching levels comparable to or even exceeding, those observed in pigs. Conversely, for the remaining antibiotics-including penicillins (AMX, AMP, PEN), aminoglycosides (GEN, NEO, STR), and cephalosporins (CED, CEC, CPN)-resistance remains notably low (below 10%). Within the tetracycline class, resistance in hens is characterized as moderate for TET (~45%) and low for OTC and CTC (~10÷25%). The antibiotic resistance profiles of *Enterococcus* strains in pig and hen populations exhibit significant multifaceted variability: certain antibiotic classes exhibit universal ineffectiveness, while others remain highly potent. According to the study results, sulfonamides (SUM, SUC) constitute the category with the highest resistance across both species, with rates ranging from 85% to 100%. Moderate resistance levels are also observed for tetracyclines; however, significant disparities exist between pigs and hens. Specifically, while pigs exhibit moderate resistance to OTC (50÷70%), the corresponding rates in hens are markedly lower (2÷8%). This identical pattern is also observed for TET and CTC. One of the most notable disparities is identified within the aminoglycoside category. *Enterococcus* strains isolated from laying hens exhibit remarkably high resistance to NEO, approaching 85%, whereas the corresponding prevalence in pigs is significantly lower (~25%). A similar trend is observed for STR, with hen-derived strains showing a higher frequency of resistance (~78%) compared to pig isolates (~65%). Conversely, regarding ERY, pig *Enterococcus* proves to be significantly more resistant (>50%) than strains from hens (~15%). On the other hand, penicillins remain the most active agents against *Enterococcus* in both farmed animal populations. Resistance to AMX, AMP, and PEN remains at negligible or zero levels, making them highly reliable therapeutic options. Similarly, low to zero resistance is recorded for the cephalosporin CPN in both farmed animal populations. The increased resistance of *E. coli* to multiple categories of antibiotics, such as penicillins, tetracyclines and sulfonamides, is consistent with the international literature and is attributed to its genetic adaptability and the presence of mobile genetic elements. The multidrug resistance of the bacterium may be directly linked to the presence of plasmids, which carry one or more resistance genes. Plasmid exchange can occur rapidly when different species coexist in environments characterized by intense microbial growth. In contrast, *Enterococcus* showed more selective resistance patterns, with particularly increased resistance to sulfonamides and, in some cases, to aminoglycosides. This widespread resistance pattern suggests that sulfonamides are widely and systematically used in these livestock farms. In addition, the use of these antibiotics may be administered at low doses over prolonged periods (e.g. as growth promoters), thereby accelerating the selection of resistant strains.

For each farm (one fecal sample per farm), antibiotic resistance testing was performed using a standardized experimental design that generated a total of 5,760 susceptibility observations per bacterial group (*Escherichia coli* or *Enterococcus spp*.). This total resulted from testing multiple bacterial isolates against 15 antibiotics, each evaluated at four different concentrations, across replicated assays. The same experimental scheme was applied to all pig and laying hen livestock farms, ensuring methodological consistency among samples. In addition, a total of 96 isolates were tested as controls under identical experimental conditions. In all samples (for both *E. coli* and *Enterococcus*), the MAR index appears to be greater than 0.2, confirming that these units are environments of high selective pressure from antibiotic. Pigs constitute a larger reservoir of multi-resistant *E. coli* strains compared to hens in these samples, with indices approaching the levels of untreated sewage (0.630). MAR values for *E. coli* range from 0.53 to 0.62. The value of 0.62 in sample I is extremely high and is in line with previous findings that record an average of 0.595 for pig farms [38,40]. The percentage of resistant strains is also very high (62.45% in sample I), which suggests that the majority of bacteria have developed resistance to multiple antibiotics simultaneously. *Enterococcus* MAR values are lower than those of *E. coli* but remain high (0.24÷0.40). An increase in the index to 0.40 (sample II) indicates a significant variation in the exposure or resistance of enterococci at this time point (Table 2). For laying hens, although the values are lower than those recorded for pigs, they remain consistently above the risk threshold. The MAR index for *E. coli* ranges between 0.22 and 0.30. These values are lower than the 0.457 average reported in the literature for laying hens, yet they still characterize the unit as a source of relative risk [40, 46].

Table 2. Antibiotic resistance profile and MAR index of *E. coli* and *Enterococcus* strains isolated from pig farm samples

		sample I	sample II	sample III
E.coli	Total number of antibiotic susceptibility tests	5760	5760	5760
	Resistant isolates	3597 (62.45%)	3221 (55.92%)	3061 (53.14%)
	MAR index	0.62	0.56	0.53
Enterococcus strains	Total number of isolates	5760	5760	5760
	Resistant isolates	1390 (24.13%)	2326 (40.38%)	1834 (31.84%)
	MAR index	0.24	0.40	0.32

The resistance rate ranges from 21% to 30%. Regarding *Enterococcus* strains, a remarkable stability in the MAR index (0.25÷0.26) is observed (Table 3). This stability is particularly worrying, as it indicates the continuous and permanent presence of multidrug-resistant enterococci in the environment of birds, which belong to the "ESKAPE" group of pathogens [47,48].

Table 3. Antibiotic resistance profile and MAR index of *E. coli* and *Enterococcus* strains isolated from laying hens' samples

		sample I	sample II	sample III
E.coli	Total number of antibiotic susceptibility tests	5760	5760	5760
	Resistant isolates	1257 (21.82%)	1724 (29.93%)	1523 (26.44%)
	MAR index	0.22	0.30	0.26
Enterococcus strains	Total number of isolates	5760	5760	5760
	Resistant isolates	1443 (25.05%)	1441 (25.02%)	1505 (26.13%)
	MAR index	0.25	0.25	0.26

Overall, the findings strongly suggest that antimicrobial resistance in the area represents an established and entrenched characteristic, arising from farm management practices and the prolonged exposure of bacterial populations to subtherapeutic antibiotic concentrations. Similar observations have been reported in previous studies. For instance, research involving the genera *Pseudomonas* and *Klebsiella* reported a high MAR index value of 0.4, emphasizing the importance of systematic monitoring of antimicrobial resistance patterns [37]. Nevertheless, despite the utility of the MAR index as a rapid risk assessment tool, potential confounding factors—such as environmental conditions and the coexistence of non-resistant strains—should also be taken into consideration when evaluating public health implications. The present study did not include direct recording of antibiotic administration practices at the investigated farms. Therefore, no specific information is available regarding the exact antibiotics used, dosage schemes, duration of administration, or therapeutic purposes. The observed resistance patterns and elevated MAR index values indirectly suggest frequent or prolonged exposure of bacterial populations to antimicrobial agents commonly used in livestock production, particularly tetracyclines, sulfonamides, penicillins, and macrolides. However, these conclusions are based on phenotypic resistance profiles rather than documented farm-level antibiotic usage records.

Cluster analysis, in combination with the source data, provides a clear understanding of how antibiotic

use in livestock farms shapes the "microbial footprint" of each species. Furthermore, dividing the dendrogram into two major clusters indicates that antimicrobial resistance is primarily influenced by two basic elements (a) the species of bacteria, i.e. whether the bacteria is *E. coli* or *Enterococcus* and (b) the management practices of farmed animals, including the management of antibiotics and the overall care provided in each livestock farm. The internal consistency of the measurements is evident, as samples I, II, and III within each category are clustered closely together (Figure 4). This indicates that antimicrobial resistance is not a random occurrence but rather a stable characteristic of the bacterial population within each farm. Notably, the *E. coli* samples from pigs (Pig_Ecoli) form a completely distinct main cluster on the left side of the dendrogram.

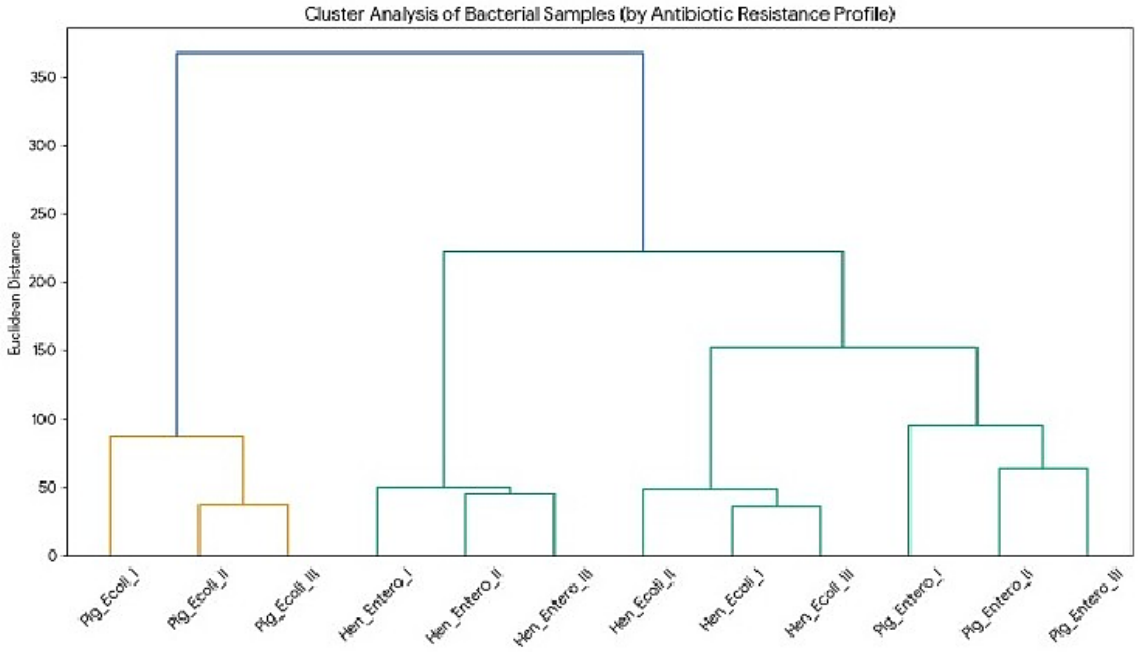

Fig. 4. Dendrogram of hierarchical cluster analysis based on antimicrobial resistance profiles of *E.coli* and *Enterococci* from pig and laying hen farms

Their pronounced separation from the remaining samples suggests an exceptionally high and broad resistance profile. As shown in the graphs, *E. coli* isolates from pigs exhibit extremely high resistance rates-often exceeding 80÷90%—to antibiotics such as AMP, AMX, ERY, and TET. The enterococci samples from laying hens (Hen_Entero) also cluster closely together, displaying a distinct resistance pattern characterized by high resistance to NEO, sulfonamides (SUM/SUC), and STR. Notably, in the dendrogram, *E. coli* isolates from laying hens (Hen_Ecoli) appear to be more closely related to the enterococci samples (Pig_Entero and Hen_Entero) than to the *E. coli* isolates from pigs. This observation suggests that husbandry practices and antibiotic usage in laying hens impose a selective pressure environment that differs substantially from that observed in pig production systems.

CONCLUSIONS

This study, highlighting the breadth of the sample, the large number of antimicrobial agents and the application of combinatorial statistical tools, enhanced the reliability of the data and allowed the interpretation of the results beyond a simple descriptive approach.

The results indicate the need to carefully examine the use of antibiotics in animals due to their critical importance for both human and animal health, thereby influencing veterinary pharmacology and public health policies. To manage this threat and limit the spread of resistant microbes through the food chain, livestock farms should follow specific practices, including (a) limiting prolonged use of antibiotics at low concentrations as growth promoters, (b) establishing systematic monitoring of resistance patterns and antibiotic consumption, using the MAR index as a cost-effective and rapid risk assessment tool, (c) implementing stricter hygiene and infection control measures to prevent

colonization and spread of multidrug-resistant pathogens, and (d) evaluating alternative antimicrobial strategies.

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