Insights into bacterial communities: multidrug-resistant and biofilm-forming bacteria in poultry droppings

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An ever-increasing demand for high-quality protein sources like poultry products, along with excessive use of antibiotics in animal husbandry are contributing factors to the growing global problem of antimicrobial resistance (AMR). The overuse of antibiotics in poultry and the dissemination of poultry faecal waste in the environment results in the propagation and spread of multidrug-resistant (MDR) bacteria. We investigated the bacterial diversity of poultry droppings by targeted amplicon sequencing and determined the resistance pattern of 165 bacterial isolates against various types of antibiotics. We found that the species of genera *Enterobacter*, *Enterococcus*, *Escherichia*, *Proteus*, *Bacillus* and *Shigella* showed resistance against beta-lactams, cephalosporins, fluoroquinolones, sulphonamide, nitrofurantoin, polymyxin E and aminoglycosides. In addition, we detected strong biofilm-producing isolates of *Enterobacter*, *Bacillus*, *Proteus*, *Escherichia* and *Enterococcus*. The detection of biofilm-forming MDR bacteria in poultry droppings highlights the need for proactive measures to mitigate their growth and transmission. High-throughput sequencing revealed the differential prevalence of amplicon sequence variants belonging to *Lactobacillus*, *Corynebacterium* and *Bacteroides*. Functional imputations support the observed potential of biosynthesis of divergent antibiotics and drug resistance. Our findings highlight that poultry droppings harbour a diverse array of antibiotic-resistant bacteria, underscoring the significance of continuous surveillance and appropriate disposal methods to counteract the escalating problem of multidrug resistance under the 'One Health' approach.

Keywords: Antibiotics, antimicrobial resistance, biofilms, multidrug-resistant bacteria, poultry droppings.

ANTIMICROBIAL RESISTANCE (AMR) is one of the most important public health issues worldwide, affecting both human and animal health. Indiscriminate usage of antibiotics in animal husbandry and unregulated ad hoc disposal of inadequately treated waste effluents in the environment are contributing factors to the local and global spread of AMR[1]. India has been one of the world's largest consumers of antibiotics, with around 5071 million defined daily doses (DDD) consumed in 2015 (refs 2, 3). An ever-increasing demand for high-quality protein products like milk, poultry, meat and fish has led to the unregulated use of antibiotics in the animal production sector in India[2]. This contributes to continuing the spread of AMR, from animal food sources to humans and the environment at large, by the farm-to-fork approach. The most widely used antibiotics in poultry feed include aminoglycosides, beta-lactams, colistin, quinolones, sulfonamides and tetracyclines[4].

The use of antibiotics in poultry feed in conventional poultry farming practices to ensure flock health, growth promotion, and prophylaxis of diseases has led to the rise in AMR worldwide[5]. Thus, the recent methods adopted by various nations under the 'One Health' approach to curbing the growing AMR throughout the world use antibiotic-free (ABF) production or alternative to antibiotics like probiotics, prebiotics, essential oils, enzymes, etc. in poultry production[6]. The overuse of antibiotics in poultry production and dissemination of poultry faecal waste by direct land dumping in the environment can result in the propagation and spread of multidrug-resistant (MDR) bacteria[7]. The biofilm formation potential of bacterial strains poses an additional health risk to humans and animals by increasing the AMR and the development of fatal infections.

The World Health Organization (WHO), Geneva, Switzerland, emphasizes surveillance studies due to the critical importance of monitoring the significant impact of AMR, often termed a silent pandemic. Considering the pivotal role of surveillance in combating AMR, the present study focuses on assesses the prevalence of antibiotics-resistant bacteria and their ability to form biofilms in conventionally raised poultry using a culture-dependent approach. Additionally, to explore the microbial communities linked to

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poultry droppings in greater detail, we integrated targeted amplicon sequencing. Previous studies have highlighted the need to employ both culture-dependent and culture-independent approaches to attain a comprehensive understanding of microbial diversity.

Materials and methods

Sample collection

Poultry dropping samples were collected in triplicate from five conventional poultry farms growing broiler chicken (JPA, JPB, JPC, JPD and PP) around Pune, Maharashtra, India. Samples were collected aseptically in sterile gamma-irradiated tubes and immediately processed for isolation and community DNA extraction.

Isolation of bacteria and identification

Bacterial isolation was performed using the serial dilution method on Mueller Hinton agar (Himedia, India) plates. Distinct bacterial colonies were purified after incubating the plates at 37°C for 48 h and further processed for molecular identification targeting the 16S rRNA gene. For molecular identification, genomic DNA extraction was performed using the standard phenol–chloroform method followed by PCR amplification of the 16S rRNA gene, as described previously[8]. The amplified products were purified and sequenced on the 3730xl Genetic Analyzer platform (Applied Biosystems, USA) available in-house[9]. The taxonomic assignment was achieved using the EzBiocloud server[10]. The 16S rRNA gene sequences have been deposited at GenBank (accession numbers OP027886–OP028050), and the pure bacterial isolates were preserved as glycerol stocks at the National Centre for Microbial Resource, National Centre for Cell Science, Pune, for further use[11].

Tests for antimicrobial susceptibility and biofilm formation

Antimicrobial susceptibility testing was performed using the disc diffusion method to determine the resistance of the bacterial isolates against 12 antibiotics mentioned in Supplementary Table 1 (Dodeca Universal II disc, HiMedia, India). The resistance and sensitivity of the bacterial isolates were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[12,13]. The isolates belonging to *Enterobacterales* showing resistance to colistin were further tested using the standard colistin agar test[12]. Further, biofilm formation of the bacterial isolates was assessed using the modified microtitre plate test in accordance with Stepanović *et al.*[14]. Biofilm formation by the isolates was tested using tryptone soy broth containing 1% glucosee. The resultant biofilms were stained using 0.1% crystal violet solution, followed by washing and air-drying. The dye bound to the cells was resolubilized with 30% glacial acetic acid, and optical density (OD) was measured at 570 nm using the SpectraMax Plus 384 Microplate reader (Molecular Devices, USA)[14].

Targeted amplicon sequencing

The community DNA from poultry dropping samples was extracted using Qiagen's QIAamp PowerFecal Pro DNA kit (Qiagen, The Netherlands). The concentration and quality of the DNA were checked using a Nanodrop One Spectrophotometer (Thermo Fischer Scientific, USA). Targeted amplification of the V4 region of the 16S rRNA gene using the specific primer set 515F and 806R was carried out. Subsequently, the library preparation and amplicon sequencing were carried out on the in-house Illumina MiSeq platform (California, USA), as described previously[15]. The raw sequences generated in this study were submitted to NCBI SRA under BioProject ID PRJNA804333.

Bioinformatics and statistical analysis

The resultant raw reads generated were trimmed and analysed until the amplicon sequence variant (ASV) using the DADA2 package v1.16 in R environment[16]. Taxonomic assignments were performed using the SILVA 138.1 database (https://www.arb-silva.de/documentation/release-1381/). Further downstream analysis was performed in R using R-packages ggplot2, phyloseq, microbiome and tidyverse. The bacterial communities associated with the poultry droppings were also examined for their potential of harbouring drug-resistance genes using the PICRUSt2 pipeline[17].

Results and discussion

According to WHO, the continuous surveillance of AMR is a vital step in preventing the drastic rise of deaths caused by it, currently estimated at 4.95 million annually[18]. The development of bacterial biofilms, triggered by quorum sensing, can be detrimental to various industries, including healthcare and food production[19]. Low to middle-income countries are particularly affected by AMR due to the overuse of antibiotics in animal production, which can then be passed on to humans. It is essential to monitor the spread of AMR-causing pathogens in order to reduce mortality rate and prevent the projected 10 million deaths predicted by 2050 (ref. 18). It is important for developing nations to shift to eco-friendly methods of utilizing alternatives to antibiotics or ABF production under the One Health approach, rather than the conventional production practices of depending on antibiotics to reduce the development of AMR, which eventually leads to increase in mortality

rates in infected humans and animals causing socio-economic losses[6].

Culture-dependent study

The 16S rRNA gene-based identification of cultured isolates revealed the presence of genera *Shigella*, *Escherichia*, *Bacillus*, *Enterobacter*, *Enterococcus*, *Staphylococcus*, *Proteus*, *Klebsiella*, *Limosilactobacillus* and *Microbacterium*, as described in the Supplementary Table 2. Members of the Gram-positive family Enterococcaceae, such as *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus hirae*, *Enterococcus durans* and *Enterococcus casseliflavus* are known for their presence in poultry[7]. The Gram-negative bacterial isolates from the poultry, viz. *Shigella* sp. and *Escherichia coli*, which commonly cause diarrhoea and urinary tract infection, were found to harbour the extended-spectrum beta-lactamases (ESBLs) of SHV-type, while *Klebsiella pneumoniae* was found to contain Amp-C type beta-lactamases and ESBLs[20,21]. These bacteria are thus considered potential pathogens due to their ability to resist antibiotics. New Delhi metallo-β-lactamase-1 (NDM-1) was first identified in *K. pneumoniae* and *E. coli*, followed by *Enterobacter cloacae* and *Proteus mirabilis* of the Enterobacteriaceae family[22]. The detection of these Gram-negative bacteria, belonging to the priority pathogens list by WHO, correlates to the emergence of AMR pathogens, possibly due to the uncontrolled use of antibiotics in poultry and other animal production sectors. Members of the Staphylococcaceae family, such as *Staphylococcus arlettae* isolates identified in this study, have also been reported in poultry from Belgium in 1984 and found to be resistant to antibiotics novobiocin and beta-lactam[23,24]. Many staphylococci, including *Staphylococcus epidermidis*, known as potential AMR pathogens, were prevalently found in chickens[25]. Further, *Bacillus* sp. identified in the present study are well known for their presence in poultry wastes[26]. The presence of these isolates depicts the enrichment of potential MDR bacteria in poultry and also highlights the associated human health risk due to their plausible transmission to different tropical levels through the consumption of contaminated meat.

Antimicrobial susceptibility testing and biofilm formation

The bacterial isolates assessed for their AMR pattern showed resistance towards various classes of antibiotics, including polymyxin E containing the last-resort antibiotic, colistin. Bacteria belonging to the phyla Pseudomonadota and Bacillota were found to follow a decreasing order of resistance towards the antibiotics. Isolates belonging to genera *Bacillus*, *Enterobacter*, *Shigella*, *Escherichia*, *Proteus*, *Klebsiella* and *Staphylococcus* showed resistance towards majority of the antibiotics. In an earlier study, we reported the presence of *Escherichia* sp. and *Klebsiella* sp., which are resistant to colistin, fluoroquinolones and third-generation cephalosporins[2]. Isolates belonging to the Bacillaceae family, such as *Bacillus cereus* and other *Bacillus* sp. were found to be resistant to beta-lactams, sulphonamide and aminoglycosides[27]. Enterococci were reported to be intrinsically resistant to amikacin, gentamicin, netilin, co-trimoxazole, ceftriaxone and cefotaxime, according to the CLSI guidelines[12]. However, we observed antibiotic susceptibility in the enterococcal strains.

Colistin agar test, performed for the isolates belonging to the Enterobacteriaceae family, showed colistin resistance among *E. cloacae*, *Escherichia fergusonii*, *Escherichia marmotae*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Shigella flexneri* (Supplementary Table 3). The increased oral administration of colistin in poultry farms for the prevention of infections and growth promotion has been reported as the driving factor for colistin resistance in members of Enterobacteriaceae like *Escherichia* sp. and *Klebsiella* sp.[28]. The presence of plasmid-mediated genes, like the mobilized colistin resistance (*mcr*) genes in the pathogenic Gram-negative bacteria and their transfer via horizontal gene transfer, have been reported for the development and spread of colistin resistance in animals, humans, tertiary care hospitals and the environment[29].

The Gram-positive bacteria lack an outer membrane and are thus intrinsically resistant to colistin. This agrees with our findings for the strains of *Bacillus, Enterococcus* and *Staphylococcus* that showed resistance towards this last-resort antibiotic at ≥4 µg/ml concentration[30]. Though colistin does not possess antibacterial activity against Gram-positive bacteria as described in previous studies[31], it has been proven to cause intensive oxidative damage by enhancing the NADH metabolism in *Bacillus* sp.[32]. Therefore, this contributes to the expansion of our understanding regarding the antibacterial mode of action of colistin, achieved through the inhibition of the respiratory chain in Gram-positive bacteria.

Additionally, poultry farms have been described as sources of antibiotic resistance genes (ARGs) of aminoglycoside and sulfonamide classes of antibiotics[33]. Due to the presence of ESBLs, resistance against cell-wall synthesis inhibitors like cefotaxime and other cephalosporin drugs has been observed in members of the family Enterobacteriaceae[34]. The presence of NDM-1 aids in the hydrolysis of penicillins, cephalosporins and carbapenems, enabling escape of bacterial strains against the action of these antibiotics[22]. *Staphylococcus arlettae* has been found to produce novel beta-lactamase bla_{ARL}, making it extensively resistant to penicillin, whereas *S. epidermidis* has been found to contain ARGs against nine antibiotic classes that might pose a serious challenge to safeguard public health[24,25].

Biofilm formation, known to protect bacteria against harsh environmental conditions and various antibiotics, is an emerging health issue. Considering this, we assessed

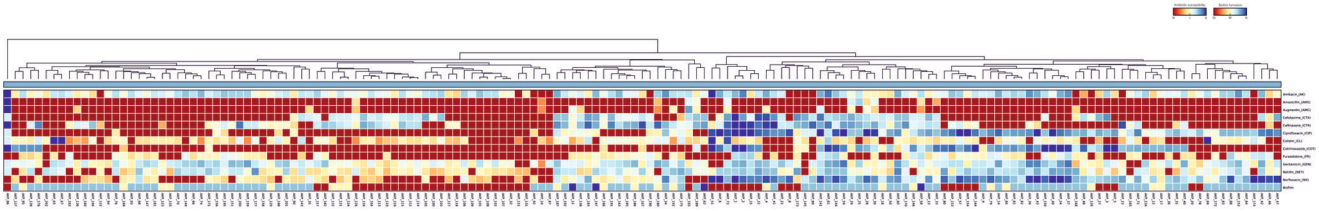

Figure 1. Heat map representing the response of bacterial isolates to various antibiotics and their biofilm formation efficiency. (For response against the antibiotics: S, Susceptible; I, Intermediate and R, Resistant. For biofilm formation: St, Strong biofilm producer; M, Moderate biofilm producer and N, No biofilm formation.) Colistin data in the heat map represent all the organisms, including bacteria known for intrinsic resistance.

biofilm formation by the bacteria isolated from poultry droppings and observed that 47 were strong biofilm producers while 21 were moderate biofilm producers. The remaining isolates showed weak biofilm formation efficiency, except for four isolates that could not form biofilms. Majority of the isolates showing biofilm formation belonged to the phyla Pseudomonadota (~70%) and Bacillota (~30%), as reported earlier by Rampadarath *et al.*[35]. The present study shows *E. cloacae*, *Proteus mirabilis*, *E. faecalis*, *K. pneumoniae*, *Escherichia* sp., *Bacillus* sp. and *Staphylococcus* sp. as strong biofilm producers, which is in agreement with earlier studies[36,37].

Biofilm formation by antibiotic-resistant bacteria in humans contributes to chronic infections, posing a serious health risk. *K. pneumoniae* strains are known to cause urinary infections, abdominal abscesses in renal transplant patients, skin abscesses and cholecystitis in hepatic transplant patients, whereas *E. cloacae* cause bacteremia in renal transplant recipients due to biofilm formation[38]. Biofilm-forming resistant strains of *E. cloacae* have also been isolated as contaminants from meat and processed food[39]. Infections caused due to biofilm-forming *Shigella* sp. are an alarming health issue across the globe. *S. epidermis*, known to form biofilms on medical devices and in nosocomial infections[40] was also detected in the poultry droppings in the present study. Figure 1 shows the MDR pathogens belonging to families Enterobacteriaceae, Enterococcaceae and Staphylococcaceae from poultry dropping samples found in the present study and their biofilm formation efficiency.

Culture-independent study

The poultry dropping samples were assessed using the 16S rRNA gene-based targeted amplicon sequencing on the Illumina MiSeq platform. The high-throughput sequencing generated a total of 107,385 paired-end reads, and after sequence denoizing and chimera removal, 60,437 reads were retained from five samples. The alpha diversity estimates based on the Shannon index showed a divergence between the samples, wherein the samples followed a descending order of diversity richness and evenness, viz. JPD > JPA > PP > JPB > JPC. In total, 12 distinct bacterial phyla were observed, of which four, viz. Bacillota (54.6%), Actinomycetota (21.1%), Pseudomonadota (17.6%) and Bacteroidota (5%) were found to be dominant in all the samples. Taxonomic profiling of the bacterial families revealed the abundance of families Enterobacteriaceae, Corynebacteriaceae, Lactobacillaceae and Enterococcaceae, which include opportunistic and obligate pathogens exhibiting multiple ARGs[5]. The significant positive association of pathogens belonging to family Enterobacteriaceae and their linked ARGs have been described and linked with aminoglycoside, tetracycline, vancomycin, phenicol and macrolide-lincosamide-streptogramin-B resistance[5]. At the genus level, we noted the enrichment of 118 unique ASVs having divergent phylogenetic relationships and abundances, wherein *Escherichia–Shigella* (16.7%) showed the maximum abundance, followed by *Corynebacterium* (14.5%) and *Ligilactobacillus* (12.2%; Figure 2 *a*). Our effort to find the common signature of these ASVs in the different samples resulted in the filtering 23 ASVs, which were differentially prevalent in the poultry droppings (Figure 2 *b*). The top five prevalent ASVs (≥80%) belonged to the genus *Ligilactobacillus*, *Lactobacillus*, *Escherichia–Shigella*, *Corynebacterium* and *Enterococcus*. These genera harbour several members considered as priority pathogens which cause life-threatening infections in humans. Further studying the shared bacterial communities by retrieving the lowest taxonomic rank (i.e. species) revealed that ASV50 is affiliated with *Lactobacillus gasseri*, a potent human pathogen. There are many diverse reports on *L. gasseri* indicating its role in bacteremia[41]. Another prevalent ASV is ASV4 affiliated with *Corynebacterium stationis*, a human pathogen isolated from an infant faecal sample. Thus, disseminating these MDR bacteria and their ARGs through horizontal gene transfer via poultry faecal waste poses a risk to public health. Collective observations from the culture dependent and independent approaches highlight that poultry droppings might act as reservoirs of antimicrobial-resistant priority pathogens harbouring various ARGs.

Functional analysis using PICRUSt2 revealed that bacterial communities contain seven major drug resistance gene families. The bacterial communities show a predominance of genes for the biosynthesis of diverse antibiotics such as streptomycin, tetracycline, ansamycins, vancomycin, penicillin and cephalosporin. Additionally, bacterial communities harbour genes for beta-lactam resistance and *Staphylococcus*

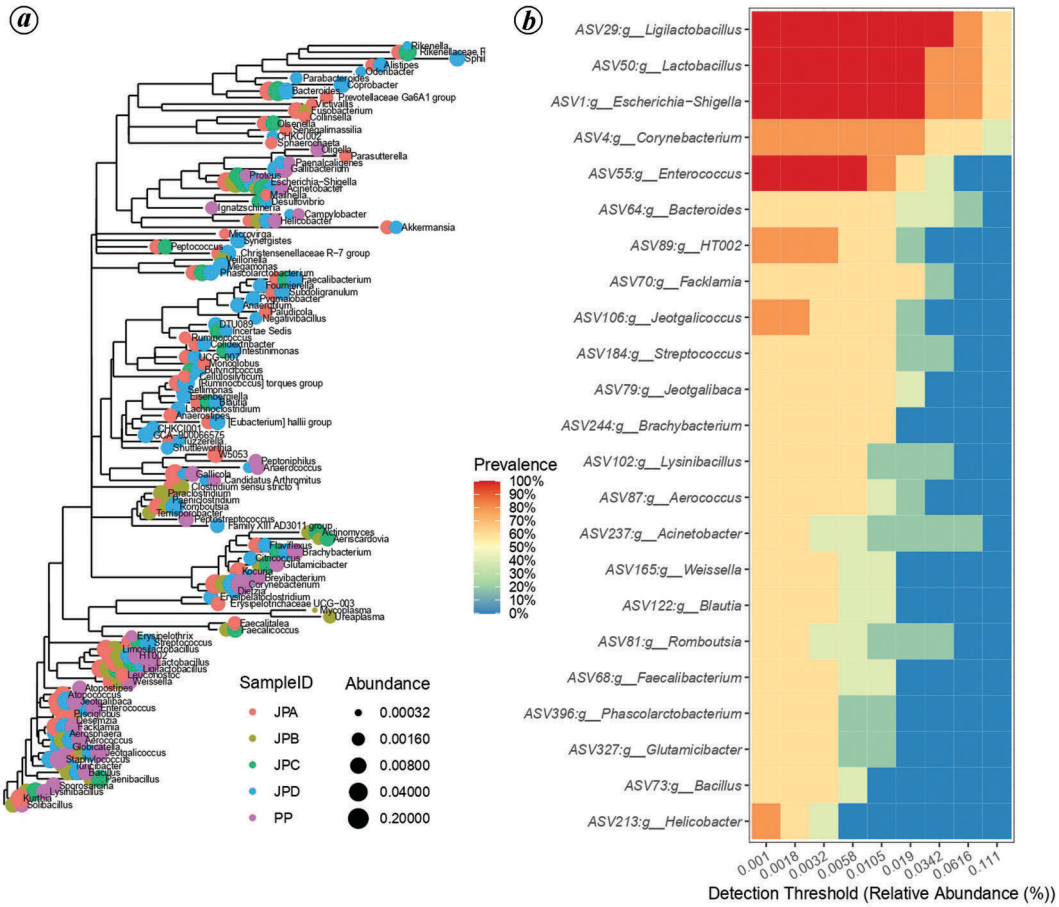

Figure 2. Bacterial community composition at the taxonomic rank of genera. ***a***, Phylogenetic distribution and the abundant bacterial genera. ***b***, Common bacterial genera found across the studied poultry droppings.

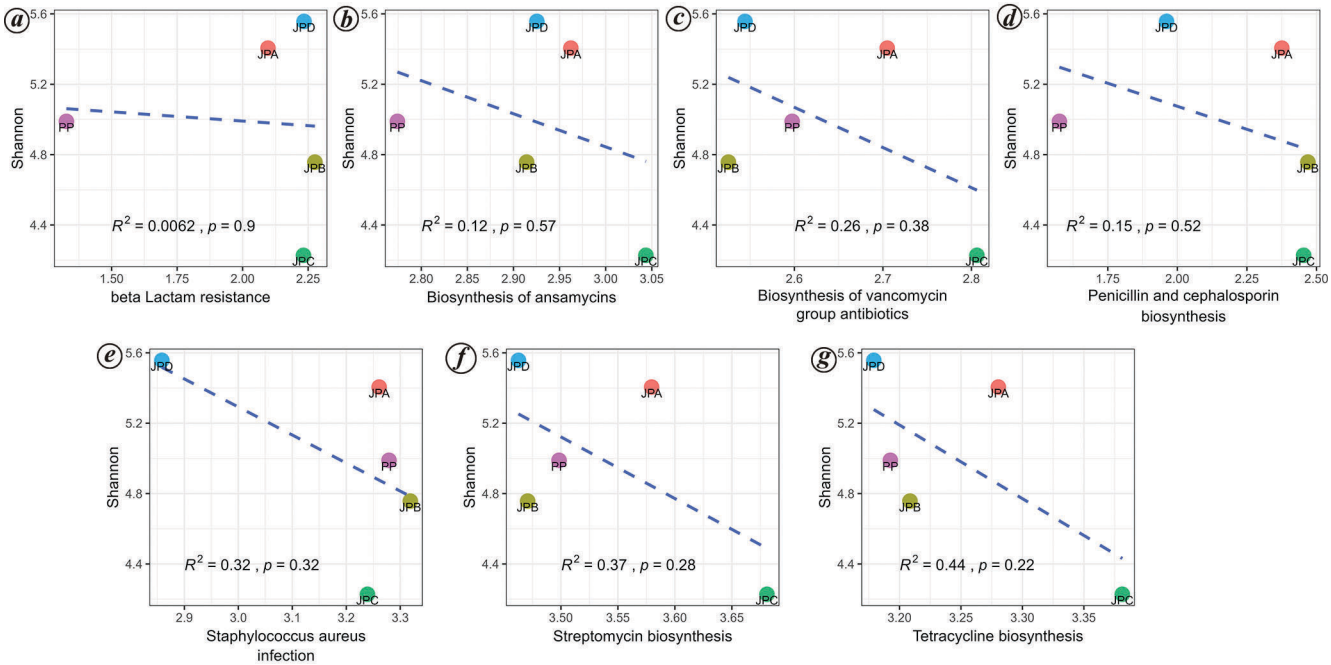

Figure 3. Relationship between bacterial diversity and the observed major drug-resistant families. The linear correlation indicates that bacterial diversity is inversely proportional to the abundance of drug resistance gene families.

aureus infection (Supplementary Figure 1 *a* and *b*). Poultry samples, viz. JPC (Shannon index = 4.22) and JPB (Shannon index = 4.75) depicting the relatively lower estimates of alpha diversity indices contained a higher abundance of the drug resistance gene families. Sample JPC contained an average 0.08-fold higher abundance for four out of seven gene families under study. Similarly, JPB harboured an average of 0.48-fold higher abundance for three out of seven gene families that included beta-lactam resistance, with penicillin and cephalosporin biosynthesis as prominent families. This observation was supported by the linear correlation indicating an inverse relationship between bacterial diversity and major drug-resistance families (Figure 3). The incidence of alteration in bacterial diversity (especially in the case of dysbiosis) and subsequent increase in the potential pathogenic microbiota have been shown by multiple studies focusing on the human and environmental microbiome[18]. We readily acknowledge the limitation of our observation of an increase in the abundance of drug-resistance genes with a decrease in bacterial diversity (Shannon index) in this study, which is based on limited samples, however the data of the current study raises serious concern mitigating the hazards to animal and public health. This study underscores the importance of proper monitoring and disposal of antibiotics used in poultry operations to prevent the spread of MDR bacteria. Alarmingly, some strains have even developed resistance to colistin. To improve infection control and mitigate further dissemination of antibiotics-resistant microorganisms, further studies are needed regarding production facilities and experimental poultry houses. In alignment with the 'One Health' approach for food safety and nutrition, this study suggests that regulating antibiotics usage in poultry production and conducting routine surveillance of these resistant pathogens are crucial. Also, adopting other methods of poultry production that replace antibiotics with alternatives or do not use them at all (ABF) is of utmost importance to win the battle against the rising AMR worldwide.

Conflict of interest: None.

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ACKNOWLEDGEMENTS. This work was supported by the Department of Biotechnology, Government of India (Grant No. BT/Coord.II/01/03/2016), under the project 'Establishment of Centre for Excellence', National Centre for Microbial Resource, National Centre for Cell Science, Pune.

Received 11 September 2023; revised accepted 26 November 2023

doi: 10.18520/cs/v126/i5/562-568