Antimicrobial resistance in ESBL and indicator *E. coli*, *Campylobacter* spp., *Salmonella* spp., methicillin-resistant *Staphylococcus aureus* (MRSA) and *Enterococcus faecalis* and *faecium* isolated from food and food-producing animals (primary production) in 2023

2023 Report

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Sciensano

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Abstract

In Belgium, the FASFC monitors the evolution of antimicrobial resistance (AMR) in food and food-producing animals (primary production). Resistance in the zoonotic bacteria *Salmonella* spp. and *Campylobacter* spp. and in methicillin-resistant *Staphylococcus aureus* (MRSA) as well as resistance in indicator bacteria *Escherichia coli*, *Enterococcus faecalis* and *Enterococcus faecium* was monitored in 2023. Moreover, a specific monitoring of presumptive extended spectrum β-lactamases/AmpC/carbapenemase producing *E. coli* is done on strains isolated from food-producing animals and meat derived thereof. Microbiological resistance was assessed using epidemiological cut-off values (ECOFF) according to EUCAST (European Committee on Antimicrobial Susceptibility Testing).

In 2023, the European Commission Implementing Decision 2020/1729 of the 17th of November 2020 was applicable for the determination of the epidemiological cut-off values as well as for the selection of antimicrobial panels (a.o. EUVSEC3/EUCAMP3).

In *Campylobacter jejuni* isolated from poultry meat, the predominant resistance profiles included ciprofloxacin combined with tetracycline. However, resistance to tetracycline was lower in 2023 (42.9%) than in 2022 (54.3%). Resistance to ertapenem in *C. jejuni* increased in 2022 (13.0%) and again in 2023 (14.3%) in comparison with 2021 (11.1%) when the antibiotic was added to the test panel. Multidrug resistance in *C. jejuni* decreased to 10.2% in 2023 compared to 15.2% in 2022. In *Campylobacter coli* isolated from poultry meat, while ciprofloxacin resistance decreased in 2023, resistance to erythromycin, ertapenem and tetracycline increased, thus increasing the multidrug resistance rate as well from 39,1% in 2022 to 48.6%.
In primary production, the monitoring of *Campylobacter coli* in fattening pigs showed overall stable resistance levels in comparison with 2022 except for a decreased resistance to ciprofloxacin (from 45.7% to 40.8%) and to erythromycin (from 19.5% to 10.9%). However, in *C. coli* isolated from veal calves, resistances to ciprofloxacin, ertapenem, gentamicin and tetracycline were lower in 2023 than in 2022 but resistances to chloramphenicol and erythromycin were higher. In *C. jejuni*, resistance to ertapenem keeps increasing since its addition in 2021. Resistances to gentamicin and tetracycline increased in 2023 as well. However, resistances to ciprofloxacin, erythromycin and chloramphenicol decreased in 2023. Overall, as seen in previous reports, antimicrobial resistance levels are lower in *C. jejuni* than in *C. coli*.

In 2023, *Salmonella* spp. recovered from food matrices have been analysed by whole genome sequencing (WGS). The highest levels of predicted antimicrobial resistance considering all serotypes and all matrices are to sulfamethoxazole (57%), followed by ampicillin (48%), tetracycline (43%) and trimethoprim (30%). Resistance to (fluoro)-quinolones associated to the *qnrB* gene was mainly predicted in isolates from broiler neck skin (11.2%) all belonging to serotype Chester (ST1954). Two isolates showed a predicted resistance to 3rd generation cephalosporins, one isolate from sunflower seeds belonging to the serotype Kentucky (ST198). This isolate harbored a $bla_{\text{CTX-M-14b}}$ together with the following genes: *aac(3)-Id*; *aac(6')-Iaa*, *aadA7*; *aph(3'')-Ib*, *aph(3')-Ia*; *aph(6)-Id*; *sul1*; *tet(A)* which confer a multidrug resistant profile including sulfamethoxazole, tetracycline and gentamicin among other aminoglycosides.
The second isolate harboring a gene conferring resistance to extended spectrum β-lactams was isolated from cut poultry meat and belonged to the serotype Infantis (ST8662). The isolate harbored the following genes: $bla_{\text{CTX-M-3}}$, *aac(6')-Iaa; aadA1; aadA1; dfrA14; sul1; tet(A)* conferring a multi drug resistance profile including extended spectrum β-lactams, aminoglycosides, trimethoprim, sulfonamides and tetracyclines.

Resistance to colistin is rare, and was only predicted in one isolate from broiler neck skin. The isolate belonged to *Salmonella* Paratyphi B var. Java (ST28) and harbored the mobile colistin resistance gene *mcr-9.1.* The isolate carried the following genes as well*, aac(6')-Iaa; dfrA1; formA; lnu(F); aph(3')-Ia; sul1; and qacE* conferring a multidrug resistant profile including trimethoprim, fosfomycin, lincosamide, aminoglycosides, sulfonamides, and quaternary ammonium biocides.

In *Salmonella* spp. isolated from food-producing animals, the most prevalent serotypes found in samples from pig caecal content were Monophasic Typhimurium (31%), Typhimurium (26%) and Derby (20%). Resistances to all antimicrobials but trimethoprim and amikacin, which is detected for the first time in this matrix, decreased in 2023. In the 3 *Salmonella* spp. isolates retrieved from veal calves caecal content, 2 belonged to the serovar Typhimurium and the last one to its monophasic variant. In both matrices, no resistance to 3rd generation cephalosporins, colistin or meropenem was detected in 2023.

In 2023, all *Salmonella* spp. isolates from fattening pigs and bovines were analysed by WGS as well. Considering all serotypes, the resistance pattern predicted for isolates from fattening pigs were as follows: tetracycline (53.50%), ampicillin (46.51%), sulfamethoxazole (44.19%) and trimethoprim (27.91%). The gene *aac(6')-Iaa* was found in all genomes independently of the serovars. This gene encodes for a chromosomal aminoglycoside acetyltransferase which confers resistance to aminoglycosides. However, *aac(6')-Iaa* and similar genes usually are transcriptionally silent and rarely become transcriptionally active. The mere presence of this gene does not confer aminoglycoside resistance in *Salmonella.* All but two isolates belonging to the serovar Typhimurium and its variant monophasic harbored the following resistance genes $bla_{\text{TEM-1B}}$, *tet (B or M) and sul (1 or 3)* conferring resistance to penicillins, tetracyclines and sulfamides. Other serotypes found in fattening pigs, such as *S*. Derby and *S*. Rissen were predicted to be occasionally resistant to the above mentioned antimicrobials and not any other resistance was predicted.
None of the isolates from either category of food-producing animals were predicted to be resistant to 3rd generation cephalosporins, ciprofloxacin or colistin. No known associated genes to the mentioned antimicrobials were found in any of the isolates.

The specific monitoring of ESBL, AmpC and carbapenemase producing *E.coli* was performed in broilers, turkeys, fattening pigs and bovines at slaughterhouse and in meat derived from these 4 categories of food producing animals at retail. In 2023, the highest prevalence of ESBL *E.coli* was found in caecal content from bovines (66.60%) followed by broilers (61.72%) and fattening pigs (29.26%). We have observed a significant decrease in the prevalence of ESBL *E. coli* isolated from broilers and fresh meat. As reported in previous years, the prevalence of ESBL *E. coli* on broiler fresh meat was the highest (45.51%) among the fresh meat categories followed by bovine fresh meat (3.19%) and pig meat (1.92%) which remains low. No meropenem-resistant isolates were detected in 2023. However, ESBL *E.coli* isolated from these matrices showed extremely high levels of multidrug resistance (>80%).
The most frequent determinants encoding for ESBL enzymes found in isolates from broilers at slaughterhouse and fresh poultry meat were $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-55}}$ and $bla_{\text{TEM-52}}$. Isolates from bovines and fattening pigs carried genes encoding for enzyme production CTX-M group 1. In addition, a significant number of isolates from bovines carried the gene $bla_{\text{CTX-M-2}}$, suggesting this category of food producing animals as a reservoir of this particular mechanism of resistance.

In indicator *E.coli*, in comparison with 2022 an overall decreased resistance to most antibiotics was detected in food-producing animals: in broilers, veal calves of less than one year and fattening pigs sampled at the slaughterhouse as well as in laying hens, breeding hens and meat bovines of less than 7 months old sampled at the farm. The highest prevalences to the critically important antimicrobials 3rd generation cephalosporins and (fluoro)quinolones were detected in *E. coli* from broiler caecal content sampled at the slaughterhouse but were lower in 2023 than in previous years. Low or very low rates of

resistance to amikacin were detected in *E. coli* isolated from pig caecal content every year since its addition in 2021. And low colistin resistance was also detected continuously over the previous years in *E. coli* isolated from veal calves caecal content. At the level of the farm, the highest resistance to (fluoro)quinolones was found in *E. coli* isolated from breeding hens while resistance to 3rd generation cephalosporins was detected in 3 *E. coli* isolates from fecal samples of bovine animals, and in one isolate from breeding hens and in one isolate from laying hens. No resistance to meropenem was detected in any of the indicator *E. coli* isolates.

Monitoring of methicillin-resistant *Staphylococcus aureus* (MRSA) was carried out in broilers, laying hens and fattening turkeys on farm in 2023. The bovines and pigs were monitored in 2021 and 2022, respectively (3 years-rotation). The aim of this monitoring is to assess the MRSA prevalence in these animal categories and determine the genotypes (STs and *spa*-types) of the collected MRSA isolates together with their AMR and virulence genes. In 2023, the MRSA prevalence was very low in broilers (1.0%) and moderate in fattening turkeys (18.5%) while null in laying hens. The MRSA prevalence remains stable since 2011 in broiler and laying hens and compared to 2020 (first year of turkey monitoring) in fattening turkeys.
In 2023, all isolates (n=1 from broilers, n=5 from fattening turkeys) were genotyped as LA-MRSA according to their STs/*spa*-types combinations. All MRSA isolated in 2023 were harboring the *mecA* gene and the *tet(M)* tetracycline resistance gene, which are also characteristics of LA-MRSA. Several other resistance genes were observed and detailed in the results. All the 6 MRSA isolates were genetically multi-drug resistant (i.e., carrying genes conferring resistance to at least 3 different antibiotic classes). However, in 2023, no gene encoding resistance to the critically important antibiotics (linezolid and vancomycin) was detected. Moreover, several virulence genes associated with the human immune evasion cluster (*sak*, *scn*), associated with toxins (*hlgA*, *hlgB*, *hlgC* and *selw*) and/or exoenzymes (*aur*) were detected among the 6 MRSA isolates analysed in 2023. One LA-MRSA isolate from fattening turkeys carried the *sak* and *scn* genes associated with the human immune evasion cluster and several genes associated with toxins (*hlgA*, *hlgB*, *hlgC*, and *selw*) and exoenzyme (*aur*). This isolate would probably not have been recently transmitted from humans to turkeys, given the livestock-associated genetic background and the carriage of the *tet(M)* gene. The genes associated with the immune evasion cluster were not observed in the other isolates. The presence of MRSA in food-producing animals and their carriage of several AMR and virulence genes represents a public health risk.

The monitoring of *Enterococcus faecalis* and *Enterococcus faecium*, organized in Belgium in food-producing animals between 2011 and 2013, and resumed in 2019, continued. Investigation of the AMR prevalence in these commensal indicator bacteria was assessed in order to complete the picture of the situation of antimicrobial resistance within our farms and slaughterhouses. Enterococci are also considered to be reservoirs of antibiotic resistance genes, present in both humans and animals. In 2023, the prevalences of enterococci species by animal category were similar to those observed in previous years. Indeed, *Enterococcus faecium* was more frequently isolated than *Enterococcus faecalis* within the samples of breeding hens (88.4%), laying hens (72.0%), veal calves (54.8%) and pigs (62.3%). Conversely, *E. faecalis* was isolated more frequently than *E. faecium* in broiler (68.8%) samples.
The antimicrobial susceptibility tests carried out this year showed that, in general, the resistance rates observed in *Enterococcus faecalis* and *Enterococcus faecium* within the various animal matrices studied have remained stable since 2019, with a few exceptions (significant decreases). Significant decreases in antimicrobial resistance were observed in 2023 for erythromycin in *E. faecalis* isolated from breeders, and for tetracycline in *E. faecalis* isolated from broilers in comparison to 2021. Resistance to tetracycline, erythromycin and to quinupristin/dalfopristin were still the most observed resistances, both in *E. faecalis* and *E. faecium*. Resistance to linezolid (n=16), a critical antibiotic for human health, was also observed in 2023, in 12 *E. faecalis* isolated from veal calves (n=11), pigs (n=1) and in 4 *E. faecium* isolated from pigs (n=3) and veal calves (n=1). Daptomycin resistance was low and observed in *E. faecalis* isolated

from breeders (n=1), layers (n=1) and pigs (n=1). Multidrug resistance was mainly observed in broilers and veal calves with 53.6% of multi-resistant *E. faecium* and 63.3 % of multi-resistant *E. faecalis*, respectively. No resistance to teicoplanin, tigecycline or vancomycin was observed in 2023.
In 2023, some enterococci were sequenced by WGS. This report also presents these WGS results and the detailed characterization of these strains, including the detection of *optrA* gene encoding linezolid resistance.

Taking together all results from both gram-negative (*E. coli*) and gram-positive indicators (enterococci) isolated from food-producing animals, resistance rates showed overall a decrease in 2023 in *E. coli* and a global status quo in enterococci (except 2 particular significant decreases).

Résumé

En Belgique, l'AFSCA surveille l'évolution de la résistance aux antimicrobiens (RAM), à la fois dans les aliments et chez les animaux producteurs de denrées alimentaires (production primaire). La résistance des bactéries zoonotiques *Salmonella* spp. et *Campylobacter* spp. et celle du *Staphylococcus aureus* résistant à la méticilline (MRSA) ont fait l'objet d'un monitoring en 2023, de même que la résistance des bactéries indicatrices *Escherichia coli*, *Enterococcus faecalis* et *Enterococcus faecium*. En outre, un monitoring spécifique des *E. coli* producteurs présumés de β-lactamase/AmpC/carbapénémase à spectre étendu a été effectué sur des souches isolées à partir d'animaux producteurs de denrées alimentaires et de viandes provenant de ceux-ci. La résistance microbiologique a été évaluée à l'aide de valeurs seuils épidémiologiques (ECOFF) établies par l'EUCAST (*European Committee on Antimicrobial Susceptibility Testing*).

En 2023, la décision d'exécution (UE) 2020/1729 de la Commission européenne du 17 novembre 2020 était applicable pour la détermination des valeurs seuils épidémiologiques ainsi que pour la sélection des panels antimicrobiens (e.a. EUVSEC3/EUCAMP3).

Chez les *Campylobacter jejuni* isolés à partir de viandes de volailles, les profils de résistance prédominants comprenaient la ciprofloxacine combinée à la tétracycline. Cependant, la résistance à la tétracycline était plus faible en 2023 (42.9%) qu'en 2022 (54.3%). La résistance de *C. jejuni* à l'ertapénème a augmenté en 2022 (13.0%) et encore en 2023 (14.3%) par rapport à 2021 (11.1%), quand l'antibiotique a été ajouté au panel de tests. La multirésistance de *C. jejuni* a diminué, passant ainsi de 15.2% en 2022 à 10.2% en 2023. Chez les *Campylobacter coli* isolés à partir de viandes de volailles, tandis que la résistance à la ciprofloxacine a diminué en 2023, la résistance à l'érythromycine, à l'ertapénème et à la tétracycline a augmenté, faisant ainsi grimper le taux de multirésistance à 48.6%, contre 39.1% en 2022.
Dans la production primaire, le monitoring de *Campylobacter coli* chez les porcs d'engraissement a montré des niveaux de résistance globalement stables par rapport à 2022, à l'exception d'une diminution de la résistance à la ciprofloxacine (de 45.7% à 40.8%) et à l'érythromycine (de 19.5% à 10.9%). Toutefois, chez les *C. coli* isolés à partir de veaux de boucherie, les résistances à la ciprofloxacine, à l'ertapénème, à la gentamicine et à la tétracycline étaient plus faibles en 2023 qu'en 2022, tandis que les résistances au chloramphénicol et à l'érythromycine étaient plus élevées. La résistance de *C. jejuni* à l'ertapénème continue d'augmenter depuis son ajout en 2021. Les résistances à la gentamicine et à la tétracycline ont également augmenté en 2023. Toutefois, les résistances à la ciprofloxacine, à l'érythromycine et au chloramphénicol ont diminué en 2023. Dans l'ensemble, comme l'ont montré les rapports précédents, les niveaux de résistance aux antimicrobiens sont plus faibles chez *C. jejuni* que chez *C. coli*.

En 2023, des isolats de *Salmonella* spp. provenant de matrices alimentaires ont été analysés par séquençage du génome entier (WGS). Si l'on considère tous les sérotypes et toutes les matrices, les niveaux les plus élevés de résistance prédite aux antimicrobiens concernent le sulfaméthoxazole (57%), suivi de l'ampicilline (48%), de la tétracycline (43%) et du triméthoprime (30%). La résistance aux (fluoro)quinolones associée au gène *qnrB* a été principalement prédite dans des isolats provenant de la peau de cou de poulets de chair (11.2%) appartenant tous au sérotype Chester (ST1954). Deux isolats ont montré une résistance prédite aux céphalosporines de 3e génération, l'un d'eux provenait de graines de tournesol appartenant au sérotype Kentucky (ST198). Cet isolat était porteur d'un gène *bla*$_{\text{CTX-M-14b}}$ ainsi que des gènes suivants : *aac(3)-Id* ; *aac(6')-Iaa*, *aadA7* ; *aph(3'')-Ib*, *aph(3')-Ia*; *aph(6)-Id* ; *sul1* ; *tet(A)* lui conférant un profil de multirésistance, notamment au sulfaméthoxazole, à la tétracycline et à la gentamicine parmi d'autres aminoglycosides.
Le second isolat porteur d'un gène conférant une résistance aux β-lactamases à spectre étendu provenait de viandes de volailles découpées et appartenait au sérotype Infantis (ST8662). Cet isolat

était porteur des gènes suivants : *bla*$_{\text{CTX-M-3}}$, *aac(6')-Iaa ; aadA1 ; dfrA14 ; sul1 ; tet(A)* conférant un profil de multirésistance, notamment aux β-lactamases à spectre étendu, aux aminoglycosides, au triméthoprime, aux sulfonamides et aux tétracyclines.
La résistance à la colistine est rare et n'a été prédite que dans un isolat provenant de la peau de cou de poulets de chair. L'isolat appartenait à *Salmonella* Paratyphi B var. Java (ST28) et était porteur du gène de résistance à la colistine mobile *mcr-9.1.* L'isolat était également porteur des gènes suivants : *aac(6')-Iaa ; dfrA1 ; formA ; lnu(F) ; aph(3')-Ia ; sul1* et *qacE* conférant un profil de multirésistance, notamment au triméthoprime, à la fosfomycine, ainsi qu'aux lincosamides, aux aminoglycosides, aux sulfonamides et aux biocides à base d'ammonium quaternaire.

Dans les isolats de *Salmonella* spp. provenant d'animaux producteurs de denrées alimentaires, les sérotypes les plus répandus dans les échantillons cæcaux de porcs étaient Monophasic Typhimurium (31%), Typhimurium (26%) et Derby (20%). Les résistances à tous les antimicrobiens, à l'exception du triméthoprime et de l'amikacine, détectée pour la première fois dans cette matrice, ont diminué en 2023. Parmi les 3 isolats de *Salmonella* spp. provenant d'échantillons cæcaux de veaux de boucherie, 2 appartenaient au sérovar Typhimurium et le dernier à sa variante monophasique. Dans les deux matrices, aucune résistance aux céphalosporines de 3^{e} génération, à la colistine ou au méropénème n'a été détectée en 2023.

En 2023, tous les isolats de *Salmonella* spp. provenant de porcs d'engraissement et de bovins ont également été analysés par WGS. Si l'on considère tous les sérotypes, le profil de résistance prédit pour les isolats provenant de porcs d'engraissement était le suivant : tétracycline (53.50%), ampicilline (46.51%), sulfaméthoxazole (44.19%) et triméthoprime (27.91%). Le gène *aac(6')-Iaa* a été trouvé dans tous les génomes, indépendamment des sérovars. Ce gène code pour une aminoglycoside acétyltransférase chromosomale conférant une résistance aux aminoglycosides. Toutefois, *aac(6')-Iaa* et les gènes similaires sont généralement transcriptionnellement silencieux et deviennent rarement transcriptionnellement actifs. La simple présence de ce gène ne confère pas de résistance aux aminoglycosides chez *Salmonella.* Tous les isolats sauf deux appartenant au sérovar Typhimurium et à sa variante monophasique étaient porteurs des gènes de résistance suivants : *bla*$_{\text{TEM-1B}}$, *tet (B* ou *M) et sul (1* ou *3)* conférant une résistance aux pénicillines, aux tétracyclines et aux sulfamides. Les autres sérotypes présents chez les porcs d'engraissement, tels que *S*. Derby et *S*. Rissen ont été prédits comme présentant une résistance occasionnelle aux antimicrobiens susmentionnés et aucune autre résistance n'a été prédite.
Aucun des isolats provenant de l'une ou l'autre catégorie d'animaux producteurs de denrées alimentaires n'a été prédit comme étant résistant aux céphalosporines de 3^{e} génération, à la ciprofloxacine ou à la colistine. Aucun gène connu associé aux antimicrobiens mentionnés n'a été trouvé dans aucun des isolats.

Le monitoring spécifique des *E. coli* producteurs de BLSE, d'AmpC et de carbapénémase a été réalisé chez les poulets de chair, les dindes, les porcs d'engraissement et les bovins à l'abattoir, ainsi que dans les viandes dérivées de ces 4 catégories d'animaux producteurs de denrées alimentaires au niveau du détail. En 2023, la plus forte prévalence d'*E. coli* BLSE a été observée dans des échantillons cæcaux de bovins (66.60%), suivi des poulets de chair (61.72%) et des porcs d'engraissement (29.26%). Nous avons observé une baisse significative de la prévalence d' *E. coli* producteurs de BLSE isolés à partir de poulets de chair et de viandes fraîches de cette catégorie d'animaux. Comme indiqué les années précédentes, la prévalence d'*E. coli* producteurs de BLSE dans la viande fraîche de poulet de chair était la plus élevée (45.51%) parmi les catégories de viandes fraîches, suivie des viandes fraîches bovines (3.19%) et porcines (1.92%), pour lesquelles la prévalence reste faible. Aucun isolat résistant au méropénème n'a été détecté en 2023. Cependant, les *E. coli* BLSE isolés dans ces matrices ont montré des niveaux de multirésistance extrêmement élevés (>80%).

Les déterminants les plus fréquents codant pour les enzymes BLSE découverts dans les isolats de poulets de chair à l'abattoir et dans les viandes fraîches de volaille étaient *bla*$_{SHV-12}$, *bla*$_{CTX-M-55}$ et *bla*$_{TEM-52}$. Les isolats provenant de bovins et de porcs d'engraissement étaient porteurs de gènes codant pour la production d'enzymes CTX-M groupe 1. En outre, un nombre important d'isolats provenant de bovins étaient porteurs du gène *bla*$_{CTX-M-2}$, ce qui suggère que cette catégorie d'animaux producteurs de denrées alimentaires constitue un réservoir de ce mécanisme de résistance particulier.

Concernant l'indicateur *E. coli*, par rapport à 2022, une baisse globale de la résistance à la plupart des antibiotiques a été détectée chez les animaux producteurs de denrées alimentaires : chez les poulets de chair, veaux de moins d'un an et porcs d'engraissement échantillonnés à l'abattoir ainsi que chez les poules pondeuses, poules reproductrices et veaux de boucherie de moins de 7 mois échantillonnés au niveau de l'exploitation. Les prévalences les plus élevées pour les antimicrobiens critiques que sont les céphalosporines de 3^{e} génération et les (fluoro)quinolones ont été détectées chez les *E. coli* isolés à partir d'échantillons cæcaux de poulets de chair prélevés à l'abattoir, mais étaient plus faibles en 2023 que les années précédentes. Des taux faibles à très faibles de résistance à l'amikacine ont été détectés chez les *E. coli* isolés à partir d'échantillons cæcaux de porcs et ce, chaque année depuis l'ajout de cette substance en 2021. Une faible résistance à la colistine a également été détectée de manière continue ces dernières années chez les *E. coli* isolés à partir d'échantillons cæcaux de veaux de boucherie.
Au niveau de l'exploitation, la résistance la plus élevée aux (fluoro)quinolones a été détectée chez les *E. coli* isolés à partir de poules reproductrices tandis qu'une résistance aux céphalosporines de 3^{e} génération a été détectée dans 3 isolats d'*E. coli* provenant d'échantillons fécaux de bovins, ainsi que dans un isolat provenant de poules reproductrices et un autre provenant de poules pondeuses. Aucune résistance au méropénème n'a été détectée dans aucun des isolats d'*E. coli* indicateurs.

En 2023, le monitoring de *Staphylococcus aureus* résistant à la méticilline (MRSA) a été effectué chez les poulets de chair, les poules pondeuses et les dindes d'engraissement au sein des exploitations. Les bovins et porcins ont fait l'objet d'un monitoring en 2021 et 2022, respectivement (rotation de 3 ans). L'objectif de ce monitoring est d'évaluer la prévalence de MRSA dans ces catégories d'animaux et de déterminer les génotypes (ST et types *spa*) des isolats de MRSA collectés, ainsi que leurs gènes de RAM et de virulence. En 2023, la prévalence de MRSA était très faible chez les poulets de chair (1.0%) et modérée chez les dindes d'engraissement (18.5%), tandis qu'elle était nulle chez les poules pondeuses. La prévalence de MRSA reste stable depuis 2011 chez les poulets de chair et les poules pondeuses. Elle reste également stable par rapport à 2020 (première année de monitoring des dindes) chez les dindes d'engraissement.
En 2023, tous les isolats (n=1 de poulets de chair, n=5 de dindes d'engraissement) ont été génotypés comme LA-MRSA selon leurs combinaisons ST/types de *spa*. En 2023, tous les isolats étaient porteurs du gène *mecA* et du gène de résistance à la tétracycline *tet(M)*, ceux-ci étant également caractéristiques des LA-MRSA. Plusieurs autres gènes de résistance ont été observés et détaillés dans les résultats. Les 6 isolats de MRSA étaient génétiquement multirésistants (c.-à-d. porteurs de gènes conférant une résistance à au moins 3 classes d'antibiotiques différentes). Toutefois, en 2023, aucun gène codant la résistance aux antibiotiques critiques (linézolide et vancomycine) n'a été détecté. En outre, plusieurs gènes de virulence associés au cluster d'évasion immunitaire humain (*sak*, *scn*), à des toxines (*hlgA*, *hlgB*, *hlgC* et *selw*) et/ou à des exoenzymes (*aur*) ont été détectés parmi les 6 isolats de MRSA analysés en 2023. Un isolat de LA-MRSA provenant de dindes d'engraissement portait les gènes *sak* et *scn* associés au cluster d'évasion immunitaire humain, ainsi que plusieurs gènes associés à des toxines (*hlgA*, *hlgB*, *hlgC*, et *selw*) et exoenzymes (*aur*). Cet isolat n'a probablement pas été récemment transmis par l'homme à la dinde, étant donné le contexte génétique associé au bétail et le portage du gène *tet(M)*. Les gènes associés au cluster d'évasion immunitaire n'ont pas été observés dans les autres isolats. La présence de MRSA chez les animaux producteurs de denrées alimentaires et le fait

que ceux-ci soient porteurs de plusieurs gènes de RAM et de virulence représente un risque pour la santé publique.

Le monitoring d'*Enterococcus faecalis* et *Enterococcus faecium* organisé en Belgique chez les animaux producteurs de denrées alimentaires entre 2011 et 2013, repris en 2019, a été poursuivi. L'étude de la prévalence de la RAM au sein de ces bactéries commensales indicatrices a été évaluée afin de compléter le tableau de la situation de la résistance aux antimicrobiens dans nos exploitations et nos abattoirs. Les entérocoques sont également considérés comme des réservoirs de gènes de résistance aux antibiotiques, présents à la fois chez l'homme et chez les animaux. En 2023, les prévalences des espèces d'entérocoques par catégorie animale étaient similaires à celles observées les années précédentes. En effet, l'espèce *Enterococcus faecium* a été plus souvent isolée que l'espèce *Enterococcus faecalis* dans les échantillons de poules reproductrices (88.4%), de poules pondeuses (72.0%), de veaux de boucherie (54.8%) et de porcs (62.3%). Inversement, l'espèce *E. faecalis* a été isolée plus souvent que l'espèce *E. faecium* dans les échantillons de poulets de chair (68.8%).
Les tests de susceptibilité antimicrobienne effectués cette année ont montré qu'en général, les taux de résistance observés chez *Enterococcus faecalis* et *Enterococcus faecium* dans les différentes matrices animales étudiées sont stables depuis 2019, à quelques exceptions près (baisses significatives). En 2023, des baisses significatives de la résistance aux antimicrobiens ont été observées par rapport à 2021 pour l'érythromycine chez *E. faecalis* isolés à partir de poules reproductrices et pour la tétracycline chez *E. faecalis* isolés à partir de poulets de chair. Les résistances à la tétracycline, à l'érythromycine et à la quinupristine/dalfopristine étaient toujours les résistances les plus observées, tant chez *E. faecalis* que chez *E. faecium*. La résistance au linézolide (n=16), un antibiotique critique pour la santé humaine, a également été observée en 2023 dans 12 *E. faecalis* provenant de veaux de boucherie (n=11) et de porcs (n=1) et dans 4 *E. faecium* provenant de porcs (n=3) et de veaux de boucherie (n=1). La résistance à la daptomycine était faible et a été observée chez *E. faecalis* isolés à partir de poules reproductrices (n=1), de poules pondeuses (n=1) et de porcs (n=1). Une multirésistance a principalement été observée chez les poulets de chair et les veaux de boucherie, avec respectivement 53.6% d'*E. faecium* multirésistants et 63.3% d'*E. faecalis* multirésistants. Aucune résistance à la teicoplanine, la tigécycline ou la vancomycine n'a été observée en 2023.
En 2023, certains entérocoques ont été séquencés par séquençage du génome entier (WGS). Ce rapport présente également les résultats du WGS et la caractérisation détaillée de ces souches, y compris la détection du gène *optrA* codant la résistance au linézolide.

Si l'on considère l'ensemble des résultats obtenus en 2023 pour les indicateurs à Gram aussi bien négatif (*E. coli*) que positif (entérocoques) isolés à partir d'animaux producteurs de denrées alimentaires, on constate globalement une baisse des taux de résistance chez *E. coli* par rapport à 2022 et un statu quo chez les entérocoques (à l'exception de 2 baisses significatives particulières).

Abstract

In België volgt het FAVV de evolutie van antimicrobiële resistentie (AMR) op in voeding en voedselproducerende dieren (primaire productie). In 2023 werd de resistentie in de zoönotische bacteriën *Salmonella* spp. en *Campylobacter* spp. en in meticillineresistente *Staphylococcus aureus* (MRSA), evenals de resistentie in de indicatorbacteriën *Escherichia coli*, *Enterococcus faecalis* en *Enterococcus faecium* gemonitord. Bovendien wordt een specifieke monitoring van vermoedelijke Extended spectrum β-lactamasen/AmpC/carbapenemaseproducerende *E. coli* uitgevoerd op stammen geïsoleerd uit voedselproducerende dieren en daarvan afgeleid vlees. Microbiologische resistentie werd beoordeeld met behulp van epidemiologische cut-offwaarden (ECOFF) volgens EUCAST (European Committee on Antimicrobial Susceptibility Testing).

In 2023 was het Uitvoeringsbesluit 2020/1729 van de Europese Commissie van 17 november 2020 van toepassing voor het bepalen van de epidemiologische cut-offwaarden en voor de selectie van antimicrobiële panels (o.a. EUVSEC3/EUCAMP3).

In *Campylobacter jejuni* geïsoleerd uit pluimveevlees hadden de vaakst voorkomende resistentieprofielen betrekking op ciprofloxacine in combinatie met tetracycline. De resistentie tegen tetracycline was evenwel lager in 2023 (42.9%) dan in 2022 (54.3%). De resistentie tegen ertapenem in *C. jejuni* steeg in 2022 (13.0%), en nam ook toe 2023 (14.3 %) in vergelijking met 2021 (11.1%), het jaar waarin het antibioticum aan het testpanel werd toegevoegd. Multiresistentie tegen geneesmiddelen in *C. jejuni* daalde in 2023 naar 10.2%, in vergelijking met de 15.2% van 2022. Bij *Campylobacter coli* geïsoleerd uit pluimveevlees nam de resistentie tegen ciprofloxacine in 2023 af, maar nam de resistentie tegen erytromycine, ertapenem en tetracycline toe, waardoor ook de multiresistentie tegen geneesmiddelen steeg van 39.1% in 2022 naar 48.6%.
De monitoring van *Campylobacter coli* in vleesvarkens uit de primaire productie gaf over het algemeen stabiele resistentieniveaus in vergelijking met 2022, met uitzondering van een verlaagde resistentie tegen ciprofloxacine (van 45.7% naar 40.8%) en tegen erytromycine (van 19.5% naar 10.9%). In *C. coli* geïsoleerd uit vleeskalveren was de resistentie tegen ciprofloxacine, ertapenem, gentamicine en tetracycline echter lager in 2023 dan in 2022, maar de resistentie tegen chlooramfenicol en erytromycine was hoger. De resistentie tegen ertapenem in *C. jejuni* blijft stijgen sinds de toevoeging ervan in 2021. Ook de resistentie tegen gentamicine en tetracycline steeg in 2023. De resistentie tegen ciprofloxacine, erytromycine en chlooramfenicol daarentegen daalde in 2023. Over het algemeen zijn, zoals in eerdere rapporten vermeld, de antimicrobiële resistentieniveaus lager bij *C. jejuni* dan bij *C. coli*.

In 2023 werd *Salmonella* spp. uit voedselmatrices geanalyseerd met behulp van whole genome sequencing (WGS). Over alle serotypen en matrices heen worden de hoogste niveaus van antimicrobiële resistentie voorspeld tegen sulfamethoxazol (57%), en daarna tegen ampicilline (48%), tetracycline (43 %) en trimethoprim (30%). De resistentie tegen (fluoro)quinolonen, in verband gebracht met het *qnrB*-gen, werd vooral voorspeld voor isolaten uit het nekvel van braadkippen (11.2%), die allemaal tot het serotype Chester (ST1954) behoren. Twee isolaten gaven een voorspelde resistentie weer tegen cefalosporines van de 3de generatie; één isolaat uit zonnebloemzaad behoorde tot het serotype Kentucky (ST198). Dit isolaat droeg een *bla*$_{\text{CTX-M-14b}}$ samen met de volgende genen: *aac(3)-Id*; *aac(6')-Iaa*; *aadA7*; *aph(3'')-Ib*; *aph(3')-Ia*; *aph(6)-Id*; *sul1*; *tet(A)*. Die genen zorgen voor een profiel van multiresistentie tegen geneesmiddelen, waaronder sulfamethoxazol, tetracycline en gentamicine, en andere aminoglycosiden.
Het tweede isolaat met een gen dat resistentie bood tegen extended spectrum β-lactamasen werd geïsoleerd uit gesneden pluimveevlees, en behoorde tot het serotype Infantis (ST8662). Het isolaat droeg de volgende genen: bla$_{\text{CTX-M-3}}$; *aac(6')-Iaa*; *aadA1*; *dfrA14*; *sul1*; *tet(A)*. Die zorgden voor een profiel van multiresistentie tegen geneesmiddelen waaronder extended spectrum β-lactamasen, aminoglycosiden, trimethoprim, sulfonamiden en tetracyclines.

Resistentie tegen colistine is zeldzaam, en werd enkel voorspeld in één isolaat uit nekvel van braadkippen. Het isolaat behoorde tot *Salmonella* Paratyphi B var. Java (ST28) en droeg het mobiele colistineresistentiegen *mcr-9.1*. Het isolaat droeg bijkomend de volgende genen: *aac(6')-Iaa*; *dfrA1*; *formA*; *lnu(F)*; *aph(3')-Ia*; *sul1*; *qacE*. Die zorgden voor een profiel van multiresistentie tegen geneesmiddelen waaronder trimethoprim, fosfomycine, lincosamide, aminoglycosiden, sulfonamiden en quaternaire ammoniumbiociden.

In *Salmonella* spp. geïsoleerd uit voedselproducerende dieren waren de meest gangbare serotypen die in monsters van caecuminhoud van varkens werden gevonden, monofasische Typhimurium (31%), Typhimurium (26%) en Derby (20%). Resistentie tegen alle antimicrobiële stoffen, buiten trimethoprim en amikacine, dat voor het eerst in deze matrix werd gedetecteerd, daalde in 2023. Van de 3 *Salmonella* spp.-isolaten die uit caecuminhoud van vleeskalveren werden gehaald, behoorden er 2 tot het serotype Typhimurium, en het andere tot de monofasische variant. Bij geen van beide matrices werd in 2023 resistentie tegen cefalosporines van de 3de generatie, colistine of meropenem gedetecteerd.

In 2023 werden ook alle *Salmonella* spp.-isolaten uit vleesvarkens en runderen met behulp van WGS geanalyseerd. Over alle serotypen heen was dit het resistentiepatroon dat werd voorspeld voor isolaten uit vleesvarkens: tetracycline (53.50%), ampicilline (46.51%), sulfamethoxazol (44.19%) en trimethoprim (27.91%). Het gen *aac(6')-Iaa* werd in elk genoom, onafhankelijk van het serotype, teruggevonden. Dit gen codeert voor een chromosomale aminoglycoside-acetyltransferase, die resistentie verleent tegen aminoglycosiden. Desondanks vindt bij *aac(6')-Iaa* en vergelijkbare genen gewoonlijk geen transcriptie plaats; ze worden zelden transcriptioneel actief. Louter de aanwezigheid van dit gen verleent geen resistentie tegen aminoglycoside in *Salmonella*. Buiten twee isolaten die tot het serotype Typhimurium en de monofasische variant behoren, droegen ze alle de volgende resistentiegenen: *bla*$_{\text{TEM-1B}}$, *tet* (*B* of *M*) en *sul* (*1* of *3*). Die verlenen resistentie tegen penicillines, tetracyclines en sulfamiden. Voor andere serotypen die in vleesvarkens werden gevonden, zoals *S.* Derby en *S.* Rissen, werd voorspeld dat ze occasioneel resistent zouden zijn tegen de hogervermelde antimicrobiële stoffen; er werd geen andere resistentie voorspeld.
Voor geen enkele van de isolaten van een van de categorieën van voedselproducerende dieren werd voorspeld dat ze resistent waren tegen cefalosporines van de 3de generatie, ciprofloxacine of colistine. In geen enkele van de isolaten werden gekende genen in verband met de hogervermelde antimicrobiële stoffen gevonden.

De specifieke monitoring van ESBL-, AmpC- en carbapenemaseproducerende *E. coli* werd uitgevoerd voor braadkippen, kalkoenen, vleesvarkens en runderen in het slachthuis en voor vlees afkomstig van deze 4 categorieën van voedselproducerende dieren in de detailhandel. In 2023, werd de hoogste prevalentie van ESBL-*E. coli* aangetroffen in caecuminhoud van runderen (66.60%), gevolgd door braadkippen (61.72%) en vleesvarkens (29.26%). We zagen een significante daling in de prevalentie van ESBL-*E. coli* geïsoleerd uit braadkippen en vers vlees. Net zoals voorgaande jaren was de prevalentie van ESBL-*E. coli* in vers vlees van braadkippen het hoogste (45.51%) onder de categorieën vers vlees, gevolgd door vers vlees van runderen (3.19%) en varkens (1.92%), wat laag blijft. In 2023 werden geen isolaten gedetecteerd die resistent waren tegen meropenem. ESBL-*E. coli* geïsoleerd uit deze matrices vertoonde echter extreem hoge niveaus van multiresistentie tegen geneesmiddelen (> 80%).
De vaakst voorkomende determinanten die coderen voor ESBL-enzymen, die werden gevonden in isolaten van braadkippen in het slachthuis en vers pluimveevlees, waren bla$_{\text{SHV-12}}$, *bla*$_{\text{CTX-M-55}}$ en *bla*$_{\text{TEM-52}}$. Isolaten uit runderen en vleesvarkens droegen genen die coderen voor de enzymproducerende CTX-M-groep 1. Daarenboven droeg een substantieel aantal isolaten uit runderen het gen *bla*$_{\text{CTX-M-2}}$, wat suggereert dat deze categorie van voedselproducerende dieren een reservoir is voor dit specifieke resistentiemechanisme.

Wat betreft de indicator *E. coli* werd in vergelijking met 2022 een algemene daling van de resistentie tegen het merendeel van de antibiotica vastgesteld bij voedselproducerende dieren: bij braadkippen, kalveren jonger dan één jaar en vleesvarkens die werden bemonsterd in het slachthuis, alsook bij leghennen, fokhennen en vleeskalveren jonger dan 7 maanden die werden bemonsterd op het landbouwbedrijf. De hoogste prevalenties van resistentie tegen kritische antibiotica, zoals cefalosporines van de 3de generatie en (fluoro)quinolonen, werden vastgesteld bij *E. coli* geïsoleerd uit monsters van de caecuminhoud van braadkippen die werden genomen in het slachthuis, maar deze prevalenties waren lager in 2023 dan in de voorgaande jaren. Er werden lage tot zeer lage resistentieniveaus tegen amikacine vastgesteld bij *E. coli* geïsoleerd uit monsters van de caecuminhoud van varkens en dit voor elk jaar sinds de toevoeging van deze stof in 2021. De laatste jaren werd ook continu een zwakke resistentie tegen colistine vastgesteld bij *E. coli* geïsoleerd uit monsters van de caecuminhoud van vleeskalveren.
Op het niveau van het bedrijf, werd de hoogste resistentie tegen (fluoro)quinolonen vastgesteld bij *E. coli* geïsoleerd uit fokhennen, terwijl er resistentie tegen cefalosporines van de 3de generatie werd vastgesteld in 3 isolaten van *E. coli* afkomstig van fecale monsters van vleesrunderen, alsook een isolaat afkomstig van fokhennen en een ander isolaat van leghennen. In geen enkel van de indicator-*E. coli*-isolaten werd resistentie tegen meropenem vastgesteld.

In 2023 werd een monitoring van meticillineresistente *Staphylococcus aureus* (MRSA) uitgevoerd voor braadkippen, leghennen en vleeskalkoenen op de hoeve. De runderen en varkens werden respectievelijk in 2021 en 2022 (driejaarlijkse rotatie) gemonitord. Het doel van deze monitoring is de MRSA-prevalentie in deze diercategorieën te beoordelen en de genotypen (ST's en spa-typen) van de verzamelde MRSA-isolaten te bepalen, samen met hun AMR- en virulentiegenen. De MRSA-prevalentie was in 2023 zeer laag in braadkippen (1.0%) en gemiddeld in vleeskalkoenen (18.5%); bij leghennen was er geen prevalentie. De MRSA-prevalentie blijft sinds 2011 stabiel in braadkippen en leghennen; hetzelfde geldt voor vleeskalkoenen in vergelijking met 2020 (eerste jaar van monitoring van kalkoenen). In 2023 werden alle isolaten (n=1 voor braadkippen, n=5 voor vleeskalkoenen) gegenotypeerd als LA-MRSA volgens hun combinaties van ST's/spa-typen. Alle in 2023 geïsoleerde MRSA's droegen het *mecA*-gen en het *tet(M)*-tetracycline-resistentiegen, die ook kenmerkend zijn voor LA-MRSA. In de resultaten werden meerdere andere resistentiegenen waargenomen en gedetailleerd besproken. Alle 6 MRSA-isolaten waren genetisch multiresistent tegen geneesmiddelen (met andere woorden: ze droegen genen die resistentie tegen minstens 3 verschillende antibioticaklassen verlenen). In 2023 werd er echter geen gen dat resistentie codeert tegen de kritische antibiotica (linezolid en vancomycine) gedetecteerd. Daarenboven werden verscheidene virulentiegenen die in verband worden gebracht met de humane immuunevasiecluster (*sak, scn*), met toxines (*hlgA, hlgB, hlgC* en *selw*) en/of met exo-enzymen (*aur*) gedetecteerd bij de 6 MRSA-isolaten die in 2023 werden geanalyseerd. Eén LA-MRSA-isolaat uit vleeskalkoenen droeg de *sak*- en *scn*-genen, in verband gebracht met de humane immuunevasiecluster, en verschillende genen in verband gebracht met toxines (*hlgA, hlgB, hlgC* en *selw*) en het exo-enzyme (*aur*). De overdracht van mensen op kalkoenen van dit isolaat gebeurde waarschijnlijk niet recent, gezien de vee-gerelateerde genetische achtergrond ervan en het dragerschap van het *tet(M)*-gen. In de andere isolaten werden er geen genen in verband met de immuunevasiecluster waargenomen. De aanwezigheid van MRSA in voedselproducerende dieren en hun dragerschap van verschillende AMR- en virulentiegenen vormt een risico voor de volksgezondheid.

De monitoring van *Enterococcus faecalis* en *Enterococcus faecium*, georganiseerd in België bij voedselproducerende dieren tussen 2011 en 2013, en hervat in 2019, werd voortgezet. Het onderzoek naar de AMR-prevalentie in deze commensale indicatorbacteriën werd beoordeeld om het beeld van de

situatie van antimicrobiële resistentie in onze hoeves en slachthuizen te vervolledigen. Enterokokken worden ook beschouwd als reservoirs van antibioresistentiegenen, die zowel bij mensen als dieren aanwezig zijn. In 2023 waren de prevalenties van soorten enterokokken per diercategorie vergelijkbaar met die in de voorgaande jaren. *Enterococcus faecium* werd namelijk vaker geïsoleerd dan *Enterococcus faecalis* in de monsters van fokhennen (88.4%), leghennen (72.0%), vleeskalveren (54.8%) en varkens (62.3%). Daarentegen werd *E. faecalis* vaker geïsoleerd dan *E. faecium* in monsters van braadkippen (68.8%).
De antimicrobiële gevoeligheidstests die dit jaar zijn uitgevoerd, hebben aangetoond dat de resistentiepercentages die zijn waargenomen bij *Enterococcus faecalis* en *Enterococcus faecium* binnen de verschillende onderzochte dierlijke matrices sinds 2019 over het algemeen stabiel zijn gebleven, op enkele uitzonderingen na (significante dalingen). Significante dalingen in antimicrobiële resistentie werden in 2023 waargenomen voor erytromycine in *E. faecalis* geïsoleerd uit fokhennen, en voor tetracycline in *E. faecalis* geïsoleerd uit braadkippen, in vergelijking met 2021. Resistentie tegen tetracycline, erytromycine en quinupristine/dalfopristine waren nog steeds de meest waargenomen resistenties, zowel bij *E. faecalis* als bij *E. faecium*. Resistentie tegen linezolid (n=16), een kritisch antibioticum voor de menselijke gezondheid, werd ook in 2023 waargenomen, in 12 *E. faecalis* geïsoleerd uit vleeskalveren (n=11), varkens (n=1) en in 4 *E. faecium* geïsoleerd uit varkens (n=3) en vleeskalveren (n=1). Resistentie tegen daptomycine was laag, en werd teruggevonden in *E. faecalis* geïsoleerd uit fokhennen (n=1), leghennen (n=1) en varkens (n=1). Multiresistentie tegen geneesmiddelen werd voornamelijk waargenomen bij braadkippen en vleeskalveren met respectievelijk 53.6% multiresistente *E. faecium* en 63.3% multiresistente *E. faecalis*. In 2023 werd geen resistentie tegen teicoplanine, tigecycline of vancomycine waargenomen.
In 2023 werden sommige enterokokken gesequenced met behulp van WGS. Dit rapport geeft ook deze WGS-resultaten en de gedetailleerde karakterisering van deze stammen weer, met inbegrip van de detectie van het *optrA*-gen dat codeert voor linezolidresistentie.

Als we alle resultaten van zowel gram-negatieve (*E. coli*) en gram-positieve indicatoren (enterokokken) geïsoleerd uit voedselproducerende dieren samennemen, tonen de resistentiepercentages voor 2023 een globale daling in *E. coli*, en een globale status quo in enterokokken (2 bijzonder substantiële dalingen uitgezonderd).

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1. Introduction

Antimicrobial agents, including antibiotics, are substances used to kill microorganisms or to stop their growth and multiplication. They are frequently used to treat a wide variety of infectious diseases in humans and animals.

While antimicrobial resistance (AMR) is partly a natural phenomenon, in that each species of bacterium is resistant to a given type of antibiotic from the outset - which in turn defines the spectrum of activity of the antimicrobial - it is also an acquired phenomenon, encouraged by the use and misuse of antibiotics. Firstly, through use, since the simple fact of using an antibiotic will gradually encourage the reproduction of resistant specimens.

A well-known example of bacteria having acquired a resistance to several antibiotics is the extended spectrum β-lactamases (ESBL) producing bacteria, i.e., enzymes that confer resistance (or a reduction of activity) to a wide range of β-lactam antibiotics, including third generation cephalosporins.

Resistant bacteria have different means of propagation. In particular, when antimicrobial resistance develops in zoonotic bacteria in animals or in food, the effective treatment of infectious diseases in humans can be compromised.

In the food safety area, the authorities must protect the consumers against the risks linked to the food chain and define the best control options to reduce these risks. Scientists and risk assessors examine the factors that may lead to the presence of antimicrobial resistant bacteria in food and animals, in order to provide appropriate scientific advice to decision makers.

In Europe, EFSA monitors and analyses the situation regarding AMR in food and in food producing animals across the countries. It is assisted by the EFSA network responsible for collecting data on zoonoses. A European Commission Implementing Decision was implemented in 2014 to harmonise sampling, antimicrobial resistance analyses and data collection. This decision was replaced by the Commission Implementing Decision 2020/2729 of the 17 of November 2021, updating which antibiotics are used in the test panels and adding or updating the epidemiological cut-off values (ECOFF).

The aim of the monitoring is to detect antimicrobial resistance in zoonotic bacteria such as *Salmonella* spp. and *Campylobacter* spp. which are of particular interest in public health. These bacteria can contaminate food and cause food poisoning. Moreover, indicator commensal bacteria such as *E.coli* from healthy food-producing animals recovered at slaughterhouse are also part of the monitoring. These indicator bacteria can contaminate food. Their resistance levels are representative of the resistance among this population. They also reflect the extent of the selection pressure exerted by antibiotics inside of the intestinal flora and can be used as an indicator of the emergence and change of resistances. These microorganisms can also serve as a reservoir of resistance genes.

The monitoring of methicillin-resistant *Staphylococcus aureus* (MRSA) has been organized in Belgium in food-producing animals since 2011, in addition to the monitoring imposed by the European decision. It follows a 3-year cycle and includes farm samples from poultry, bovines or pigs, depending on the year. In 2023, the monitoring focused on broilers, laying hens and fattening turkeys. The aim of this monitoring is to assess the MRSA prevalence on farms and determine the genotypes (STs and *spa*-types) of up to 170 of collected MRSA isolates together with their AMR and virulence genes. The resistance present in farm animals is important to assess since exchanges of MRSA and potentially associated resistance from animals to humans, and vice versa, have been described. From a human health point of view, it is therefore in our interest to monitor emerging resistance in animals in order to establish possible links with cases of human infection with methicillin-resistant *Staphylococcus aureus*. Such investigations will

be facilitated with the availability of whole-genome sequencing data, as generated through the 2023 monitoring.

The monitoring of *Enterococcus faecalis* and *Enterococcus faecium*, organized in Belgium in food-producing animals between 2011 and 2013, and resumed in 2019, continued in 2023. Investigation of the AMR prevalence of these commensal indicator bacteria was assessed in order to complete the picture of the situation of antimicrobial resistance within our farms and slaughterhouses, in addition to the monitoring of indicator *E. coli*. Enterococci are also considered to be reservoirs of antibiotic resistance genes, present in both humans and animals. In addition, some of these *Enterococcus* species already possess (so-called intrinsic) resistance linked to the presence of specific genes: for example, resistance to quinupristin/dalfopristin (Synercid) present in *Enterococcus faecalis* or even resistance to vancomycin present in *Enterococcus gallinarum/Enterococcus casseliflavus*.

Since 2021, WGS has been implemented as part of official monitoring to investigate antimicrobial resistant bacteria isolated from food producing animals and in foodstuffs. There is worldwide interest in genomic monitoring to combat multidrug-resistant bacteria. This monitoring reveals the resistance genes which, when expressed, are indeed responsible for antibiotic resistance. With the advance of new technologies appeared the first generation of high throughput sequencing devices, commonly called WGS (Whole Genome Sequencing). These platforms can analyze tens of thousands of DNA sequences at once.

For this purpose, a flexible approach is described in the scientific report published by EFSA on technical specifications on harmonized monitoring of antimicrobial resistance in zoonotic and indicator bacteria in food-producing animals and food (EFSA Journal 2012). Particularly in the context of ESBL monitoring, a flexible approach for Member States/National Reference Laboratories (NRLs) to use WGS on a voluntary basis for the detection of ESBL/AmpC/carbapenemase-producing *E. coli* replaces panels 1 and 2 of the phenotypic antimicrobial susceptibility testing method in the monitoring of these organisms (EU 2020/1729). In 2023, ESBL *E. coli* from food producing animals, broilers, fattening pigs, bovines and fresh meat derived thereof were analysed by WGS and reported to EFSA. In addition, *Salmonella* spp. isolates from food, feed and primary production were sequenced under voluntary basis as well and analysed accordingly.

The sequencing of a selection of MRSA and enterococci was also carried out this year with the aim of investigating, typing and characterizing the antimicrobial resistance as well as the virulence within these strains. Specifically, genetically characterizing observed resistance to critical antibiotics such as vancomycin and linezolid is of great interest. Similarly, the characterization of MRSA associated with human lineages acquired in the community (CA-MRSA) or in the hospital (HA-MRSA) but isolated from animals is of great interest for public health.

2. Materials and method

2.1. ANTIMICROBIAL SUSCEPTIBILITY TESTING : PANELS AND INTERPRETATION CRITERIA

2.1.1. Panels of antimicrobials used for susceptibility testing

The reduction of susceptibility of a bacterium to a given antimicrobial is measured through minimal inhibitory concentration (MIC). The MIC is the lowest antibiotic concentration needed to inhibit the growth of a bacterium. The MIC value measuring this concentration is usually written in mg/L.
The MIC determination is performed according to the method described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standard Institute (CLSI). This method is recognised as an international reference method (standard ISO 20776-1 :2019). In 2023, the MIC of isolates was determined via the micro broth dilution method using the Sensititre device (ThermoFisher) and the following panels of antimicrobials : EUVSEC3 (first panel), EUVSEC 2 (second panel) for indicator *E.coli* and *Salmonella*, EUCAMP3 for *Campylobacter*, and EUVENC for *Enterococcus faecalis* and *Enterococcus faecium*. The interpretation of the results was based on the resistance thresholds (ECOFF) established by EUCAST, as described in the Commission Implementing Decision 2020/1729/EU.
The following tables (Table 1 to Table 7) show the antimicrobial resistance interpretation thresholds depending on the bacterium.

Table 1. Panel of antimicrobials tested (EUCAMP3) and interpretation thresholds for *Campylobacter jejuni*

Antimicrobial	Abbreviation	AMR interpretation thresholds > (mg / l)
Chloramphenicol	Chl	16
Ciprofloxacin	Cip	0,5
Ertapenem	Etp	0,5*
Erythromycin	Ery	4
Gentamicin	Gen	2
Tetracycline	Tet	1

*Official data unavailable, the value considered for Etp is >0,5 mg/l according to the EFSA guidelines for the reporting of AMR in 2023.

Table 2. Panel of antimicrobials tested (EUCAMP3) and interpretation thresholds for *Campylobacter coli*

Antimicrobial	Abbreviation	AMR interpretation thresholds > (mg / l)
Chloramphenicol	Chl	16
Ciprofloxacin	Cip	0,5
Ertapenem	Etp	0,5*
Erythromycin	Ery	8
Gentamicin	Gen	2
Tetracycline	Tet	2

* Official data unavailable, the value considered for Etp is >0,5 mg/l according to the EFSA guidelines for the reporting of AMR in 2023.

Table 3. Panel of antimicrobials tested (first panel EUVSEC3) and interpretation thresholds for *Salmonella* spp.

Antimicrobial	Abbreviation	AMR interpretation thresholds > (mg / l)
Amikacin	Ami	4
Ampicillin	Amp	8
Cefotaxime	Fot	0.5
Ceftazidime	Taz	2
Meropenem	Mer	0.125
Nalidixic Acid	Nal	8
Ciprofloxacin	Cip	0.064
Tetracycline	Tet	8
Colistin	Col	2*
Gentamicin	Gen	2
Trimethoprim	Tmp	2
Sulfamethoxazole	Smx	256*
Chloramphenicol	Chl	16
Azithromycin	Azi	16*
Tigecycline	Tig	0.5*

* Official data unavailable, the values considered for Col, Smx, Azi and Tig are in line with the EFSA guidelines for the reporting of AMR in 2023.

Table 4. Panel of antimicrobials tested (second panel EUVSEC2) and interpretation thresholds for *Salmonella* spp.

Antimicrobial	Abbreviation	AMR interpretation thresholds > (mg / l)
Cefoxitin	Fox	8
Cefepime	Fep	0.125*
Cefotaxime+clavulanic acid	Fot/Cl	0.5*
Ceftazidime+clavulanic acid	Taz/Cl	2*
Meropenem	Mer	0.125
Temocillin	Tem	16*
Imipenem	Imi	1
Ertapenem	Etp	0.06*
Cefotaxime	Fot	0.5
Ceftazidime	Taz	2

*Official data unavailable, the values considered for Fep, Fot/Cl, Taz/Cl Tem and Etp are in line with the EFSA guidelines for the reporting of AMR in 2023.

Table 5. Panel of antimicrobials tested (first panel EUVSEC3) and interpretation thresholds for indicator *E. coli*.

Antimicrobial	Abbreviation	AMR interpretation thresholds > (mg / l)
Amikacin	Ami	8
Ampicillin	Amp	8
Cefotaxime	Fot	0.25
Ceftazidime	Taz	0.5
Meropenem	Mer	0.125
Nalidixic Acid	Nal	8
Ciprofloxacin	Cip	0.064
Tetracycline	Tet	8
Colistin	Col	2
Gentamicin	Gen	2
Trimethoprim	Tmp	2

Sulfamethoxazole	Smx	64
Chloramphenicol	Chl	16
Azithromycin	Azi	16*
Tigecycline	Tig	0,5

* Official data unavailable, the value considered for Azi is >16 mg/l according to the EFSA guidelines for the reporting of AMR in 2023.

Table 6. Panel of antimicrobials tested (second panel EUVSEC2) and interpretation thresholds for indicator *E. coli*.

Antimicrobial	Abbreviation	AMR interpretation thresholds > (mg / l)
Cefoxitin	Fox	8
Cefepime	Fep	0.125
Cefotaxime+clavulanic acid	Fot/Cl	0.25
Ceftazidime+clavulanic acid	Taz/Cl	0.5
Meropenem	Mer	0.125
Temocillin	Tem	16
Imipenem	Imi	0,5
Ertapenem	Etp	0.06*
Cefotaxime	Fot	0.25
Ceftazidime	Taz	0.5

* Official data unavailable, the value considered for Etp is >0.06 (mg/l) according to the EFSA guidelines for the reporting of AMR in 2023.

Table 7. Panel of antimicrobial substances tested, minimum and maximum concentrations tested and interpretation thresholds for *Enterococcus faecalis* and *Enterococcus faecium*.

Antimicrobials	Abbreviation	ECOFF* (R>mg/l) - *E. faecalis*	ECOFF* (R>mg/l) - *E. faecium*	Minimum concentration mg/L	Maximum concentration mg/L
Ampicillin	AMP	4	4	0.5	64
Chloramphenicol	CHL	32	32	4	128
Ciprofloxacin	CIP	4	4	0.12	16
Daptomycin	DAP	4	8	0.25	32
Erythromycin	ERY	4	4	1	128
Gentamicin	GEN	64	32	8	1024
Linezolid	LZD	4	4	0.5	64
Quinupristin/dalfopristin (Synercid)	SYN	1[#]	1	0.5	64
Teicoplanin	TEI	2	2	0.5	64
Tetracycline	TET	4	4	1	128
Tigecycline	TGC	0.25	0.25	0.03	4
Vancomycin	VAN	4	4	1	128

* The ECOFF values used for enterococci are those published by EFSA in April 2021.
[#] intrinsic resistance in *Enterococcus faecalis*, a threshold of 1 mg/L has been set for data reporting.

2.1.2. EFSA Antimicrobial Susceptibility Classification Criteria

The levels of antimicrobial resistance are described according to the following criteria :

- Rare : <0,1%
- Very low : >0,1% to 1%
- Low : >1% to 10%
- Moderate : >10% to 20%
- High : >20% to 50%

- Very high : >50% to 70%
- Extremely high : >70%

These terms apply to any antimicrobial. However, the significance of a given level of resistance depends on the antimicrobial substance and its relative importance in human and veterinary medicine (EFSA Journal 2023;21(3):7867).

2.1.3. EFSA β-lactamases classification criteria

The second panel is used to accurately classify *E.coli* and *Salmonella* spp. isolates with resistance to third generation cephalosporins. The criteria were updated in 2023 (see Table 8).

Table 8. EFSA classification criteria for β-lactamase enzymes.

Case	Phenotype	Description
1	ESBL	Cefotaxime or ceftazidime > 1mg/L (R) and Cefoxitin ≤8mg/L (S) and Meropenem ≤ 0.12mg/L (S) and Cefotaxime/clavulanic acid or ceftazidime/clavulanic acid synergy
2	AmpC	Cefotaxime or ceftazidime > 1mg/L (R) and Cefoxitin > 8mg/L (R) and Meropenem ≤ 0.12mg/L (S) and No cefotaxime/clavulanic acid or ceftazidime/clavulanic acid synergy
3	ESBL+AmpC	Cefotaxime or ceftazidime > 1mg/L (R) and Cefoxitin > 8mg/L (R) and Meropenem ≤ 0.12mg/L (S) and Cefotaxime/clavulanic acid or ceftazidime/clavulanic acid synergy
4	Carbapenemases	Meropenem > 0.12mg/L (R) Needs confirmation
5	Other phenotypes	For phenotypes that are non-susceptible to CTX, CAZ, FOX or MEM and do not fall into any of the categories above (ESBL, AmpC, ESBL+AmpC and Carbapenemase), strains should be categorized as “Other phenotype”.

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2023, The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2020/2021. EFSA Journal 21(3):7867, page 226, Figure F.1, https://doi.org/10.2903/j.efsa.2023.7867.

2.1.4. Definition of multidrug resistance

The term multidrug resistance (MDR) refers to isolates with a resistance profile including at least three classes of antimicrobials. This means, for example, that resistance to ciprofloxacin and nalidixic acid represents resistance to a single class of antibiotics as well as resistance to cefotaxime and ceftazidime represents resistance to third generation cephalosporins or resistance to gentamicin and amikacin represents resistance to aminoglycosides.

2.1.5. Antimicrobial susceptibility testing data analysis

The number of antimicrobials to which a strain is resistant was counted and the percentages of resistance to each antimicrobial by animal category and bacterial species were calculated and represented graphically (Excel). The statistical analyses were performed using SAS 9.4 software. For

each animal category and each year, the proportion (p) of resistant isolates was calculated per antimicrobial with a 95% confidence interval (CI). A univariate logistic regression, in which each antimicrobial was considered separately, was carried out to determine, using odds ratios, whether the proportion of resistant strains was significantly higher in 2023 than in previous years.

2.1.6. Whole Genome Sequencing of ESBL *E. coli* and *Salmonella* spp.

In 2023, all presumptive ESBL *E. coli* isolated from food producing animals and meat derived thereof have been analysed using WGS.
In addition, all *Salmonella* spp. retrieved from food, feed and primary production were sequenced under voluntary basis and analysed according to the protocol described below.

Genomic DNA was extracted from isolates using the Maxwell cultured cell DNA kit (Promega, Wisconsin, USA). DNA purity was assessed with a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA) and concentration was measured with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Library preparation was performed with the *NEBNext Ultra™ II FS DNA Library Prep Kit for Illumina* (Illumina, San Diego, CA, USA) optimized by Eurofins, and sequencing was conducted on using Illumina NovaSeq 6000 platform using 2x150 Sequence mode.

Pre-processing of the sequencing data began with down sampling to approximately 100x coverage, based on the estimated genome size of 5,000,000 bp for *E. coli* , using the 'sample' command of seqtk v1.4 (available at https://github.com/lh3/seqtk). Read trimming was performed with Trimmomatic v0.39 using the following settings: LEADING=10, TRAILING=10, SLIDINGWINDOW=4:20, MINLEN=40, and ILLUMINACLIP=NexteraPE-PE.fa:2:30:10. The processed reads were de novo assembled using SPAdes v3.15.5 with the '--cov_cutoff' parameter set to 10 and the '--isolate' option enabled. Assembly metrics were calculated using QUAST 5.2.0, with the processed FASTQ files and assembled contigs as input, and the *E. coli* O157:H7 genome as reference (NCBI Reference Sequence: NC_002695.2). Processed reads were mapped to the assembly using Bowtie2 v2.5.1 with the '--end-to-end' and '--sensitive' options. The median depth was determined using the 'depth' command from samtools v1.17 with the '-a' option enabled.

Detection of antimicrobial resistance (AMR) mutations and genes was performed using ResFinder application v. 4.4.2; ResFinder Database v. 2.2.1; PointFinder Database v. 4.0.1.
Sequence types (STs) were determined using a blastn-based approach with the MLST scheme from the Institut Pasteur Paris BIGSdb instance (accessed on the 17th of December 2023).

All tools to perform the bioinformatics analysis are freely available for non-commercial use at https://galaxy.sciensano.be (registration required). The *E. coli/Salmonella* pipeline enables comprehensive characterization from raw FASTQ data, including pre-processing, quality control, and AMR predict resistance.

2.2. METHODOLOGY FOR METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

2.2.1. Sampling

In 2023, samples were taken of broilers, laying hens and fattening turkeys on farm. They are programmed to be taken by official veterinarians, divided over the year and over the different local control units based respectively on the number of such farms in each control unit. If different categories of animals are present on one farm, only one category is sampled. Ten nasal swabs from 10 different birds are taken on each holding and pooled to one sample. Each swab is transported in its own transportation tube. The swabs are pooled at the level of the laboratory to one sample per farm. The swabs are stored between 5°C and 25°C. A holding is positive when a suspected MRSA colony is detected and confirmed by Whole genome sequencing (WGS).

2.2.2. Isolation and identification

In 2023, the so-called 1-S isolation method was conducted according to the EURL-AR protocol version from 2018 (https://www.eurl-ar.eu/CustomerData/Files/Folders/21-protocols/430_mrsa-protocol-final-19-06-2018.pdf), excluding the selective enrichment step. Presence of MRSA is suspected based on colony morphology on Brilliance MRSA 2 agar from which a single colony is subcultured on blood agar. Presence of MRSA is confirmed using WGS (i.e., *S. aureus* species identification associated with a *mec* gene detection).

2.2.3. Confirmation and characterization by WGS

In 2023, 6 MRSA isolates were analyzed by WGS to determine their genotypes (sequence-types (ST) and *spa*-types) and also to detect their AMR and virulence genes.
DNA is extracted using the DNeasy® Blood and Tissue kit according to the manufacturer instructions for Gram-positive bacteria (Qiagen, Hilden, Germany). Isolate sequencing libraries were created using Nextera XT DNA library preparation (Illumina, San Diego, CA, USA) according to the manufacturer's instructions and subsequently sequenced using MiSeq V3 chemistry (Illumina) for the production of 2X250 bp paired-end reads. The *Staphylococcus* pipeline v0.1 of the in-house instance of the Galaxy workflow management system (https://galaxy-tag.sciensano.be/) was used for bioinformatic analysis including a.o. contamination check and species identification with the kraken2 tool (version 2.0.7). The classic MLST scheme used was retrieved from PubMLST.org, from which the ST results were obtained. The *spa*-types were assigned based on the Ridom database. The AMR detection was assessed through the *Staphylococcus* pipeline v0.1 as described for gene detection by Bogaerts *et al*. (2021) using 2 databases: the ResFinder database and the NCBI AMRFinderPlus database regularly updated in the in-house instance of the Galaxy workflow management system (https://galaxy-tag.sciensano.be/).
The default parameters were used for both tools (i.e., minimum coverage: 60%, minimum percentage of identity: 90%). The virulence gene detection was assessed with the VirulenceFinder tool. All the tools were used through the pipeline *Staphylococcus* v0.1 of the in-house galaxy instance and are regularly updated.

2.3. METHODOLOGY FOR *ENTEROCOCCUS FAECALIS* AND *ENTEROCOCCUS FAECIUM*

2.3.1. Enterococci sampling

Sampling for enterococci monitoring takes place from January to December and follows different procedures depending on the animal matrix studied. Samples taken at the slaughterhouse and at the farm by official FASFC agents as part of monitoring the antimicrobial resistance of commensal E. coli are also used for monitoring antimicrobial resistance in *Enterococcus faecium* and *faecalis*. For broiler, pig and veal calve samples taken at the slaughterhouse, 100 ml of faeces are taken from the large intestine (colon, cecum or rectum), and each sample corresponds to a different epidemiological unit. For the samplings of laying hens and breeding hens carried out on the farm, at least 20 ml of faeces are taken from the ground in different places (preferably 10 different places) using a sterile glove, and constitute a sample.

2.3.2. Isolation and identification by MALDI-TOF

To monitor enterococci, samples of faecal or caecal matter are resuspended in buffered peptone water (BPW) and directly inoculated onto Slanetz-Bartley agar and incubated at 41.5°C for 48 hours in the laboratories of the federal agency for food chain safety (FASFC) then transported to the LNR at Sciensano for identification and antimicrobial susceptibility testing. From Slanetz-Bartley agars, suspicious colonies are subcultured onto CSB plates (blood agars) and incubated at 37°C for 24 hours. The identification of the bacterial species is then carried out by MALDI-TOF from a pure colony from CSB plates.

2.3.3. Study of antimicrobial susceptibility

Antimicrobial resistance was determined using the micro-dilution method (Sensititre, Trek Diagnostic Systems, Thermofisher), according to the manufacturer's instructions (SOP/BAC/ANA/11) and the epidemiological cut-offs (ECOFFs) established by EUCAST or defined by the European Reference Laboratory for Antimicrobial Resistance (DTU) for *Enterococcus faecalis* and *Enterococcus faecium* (see Table 7). This method has been demonstrated to be in agreement with the ISO 20776-1:2019 reference method. For the study of antimicrobial susceptibility, a maximum of one *Enterococcus faecalis* and one *Enterococcus faecium* were analyzed per sample. Other species of enterococci are not tested. After subculture of a pure colony and incubation at 37°C for 24 hours, 3 to 5 colonies from blood agars are added to 10ml of sterile physiological water to obtain a solution of 0.5 McFarland. 30 µl of this suspension are inoculated into an 11 ml tube of Mueller-Hinton medium with TES and adjusted in cations. 50µl of this inoculum are then added to each well of a Sensititre® EUVENC plate via the AIM Automated Inoculation Delivery System and the plate is incubated at 35°C for 24 hours. Sensititre® plates are read with the Sensititre Vision System software which allows semi-automatic recording of the minimum inhibitory concentration (MIC) of the different antibiotics tested. The MIC is defined as the lowest concentration where no visible growth could be detected (see point 2.1). The definition of the MIC for linezolid is an exception: the MIC is defined as the concentration at which a decrease in growth of 80-90% compared to the growth observed for the positive control is observed. The determination of the MIC also follows a special rule for Gram-positive bacteria for chloramphenicol, erythromycin and tetracycline, for which the small pellets which have a size comparable to that illustrated in figure 3 of document M07 (11th edition) from CLSI should be ignored.

2.3.4. WGS

In 2023 a total of 19 enterococci were analyzed by WGS. Genomic DNA was extracted using the DNeasy® Blood and Tissue kit according to the manufacturer instructions for Gram-positive bacteria (Qiagen, Hilden, Germany). The raw data was analyzed within the Veterinary Bacteriology department of Sciensano via the use of the *Enterococcus* v1.1 pipeline, developed by the TAG (Transversal activities in Applied Genomics) department of Sciensano and accessible on Galaxy (https:// galaxy-tag.sciensano.be/). The use of the *Enterococcus* pipeline includes rough fragment end trimming (Trimmomatic v0.38), assembly (SPAdes v3.13.0), MLST typing, resistance gene detection, mutation detection (PointFinder) and the detection of virulence genes (VirulenceFinder), depending on the bacterial species selected (*Enterococcus faecalis* or *Enterococcus faecium*). Detection of antimicrobial resistance genes was performed as described by Bogaerts *et al.* (2021) and based on the use of 2 databases: the ResFinder database and the NCBI AMRFinderPlus database. In order to determine whether strains characterized by the same ST were phylogenetically close, comparisons of their cgMLST profiles were carried out using the MLST Phylogeny v0.1 tool.

3. Results and discussion

Results of the monitoring of antimicrobial resistance of the bacteria *Salmonella* spp., *Campylobacter jejuni*, *Campylobacter coli,* commensal indicator and ESBL producing *Escherichia coli* (*E. coli*), Methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis* and *Enterococcus faecium* indicator commensals in 2023 are presented below. Firstly, monitoring data in food products is presented, followed by monitoring in food-producing animal populations (primary production).

3.1. ANTIMICROBIAL RESISTANCE MONITORING IN ZOONOTIC AND COMMENSAL BACTERIA IN FOOD PRODUCTS.

A summary table (Table 9) shows the number of strains isolated from food samples analysed by antimicrobial susceptibility testing (AST) or whole genome sequencing (WGS).

Table 9. Number of isolates tested for antimicrobial resistance per matrix in 2023 (food)

Matrix	Number of isolates tested
Salmonella FOOD	
WGS	174
Salmonella FEED	
WGS	46
ESBL *E. coli* ESBL fresh beef meat (distribution)	
WGS	6
ESBL *E. coli* fresh pig meat (distribution)	
WGS	6
ESBL *E. coli* fresh broiler meat (distribution)	
WGS	134
ESBL *E. coli* fresh broiler meat (border control post)	
WGS	3
ESBL *E. coli* fresh turkey meat	
WGS	23
Campylobacter FOOD	
AST *C. jejuni*	49
AST *C. coli*	35

3.1.1. Antimicrobial resistance in *Campylobacter* spp.

In 2023, the NRL received 84 *Campylobacter* spp. isolates from samples of poultry meat. The species identification was done by MALDI-TOF mass spectrometry in FASFC laboratories. The minimal inhibitory concentration (MIC) was determined on 84 isolates and the results are shown in Table 10.

Table 10. Species identification of *Campylobacter* spp. tested for AMR in 2023

Species	Number
Campylobacter coli	35
Campylobacter jejuni	49
Campylobacter lari	0
Total	**84**

The MIC of *C. jejuni* (Figure 1) and *C. coli* was determined according to the method described in the Commission Implementing Decision 2020/1729 (Official Journal of the European Union 19.11.2020). The results were interpreted in accordance with the thresholds published in the Decision 2020/1729 (Annex part A, Table 3).
Table 11 lists the number of *Campylobacter jejuni* and *Campylobacter coli* isolates and their matrices of origin.

Table 11. Number of *Campylobacter jejuni* and *Campylobacter coli* isolates tested for antimicrobial susceptibility in 2023 per matrix of origin.

Technical Sheet	Description	Number	
		C. jejuni	***C. coli***
TRA 100	Fresh meat from poultry with skin	30	26
DIS 815	Poultry meat preparation	0	2
DIS 819	Fresh meat from broilers	17	7
DIS 821	Meat preparation from broilers	2	0

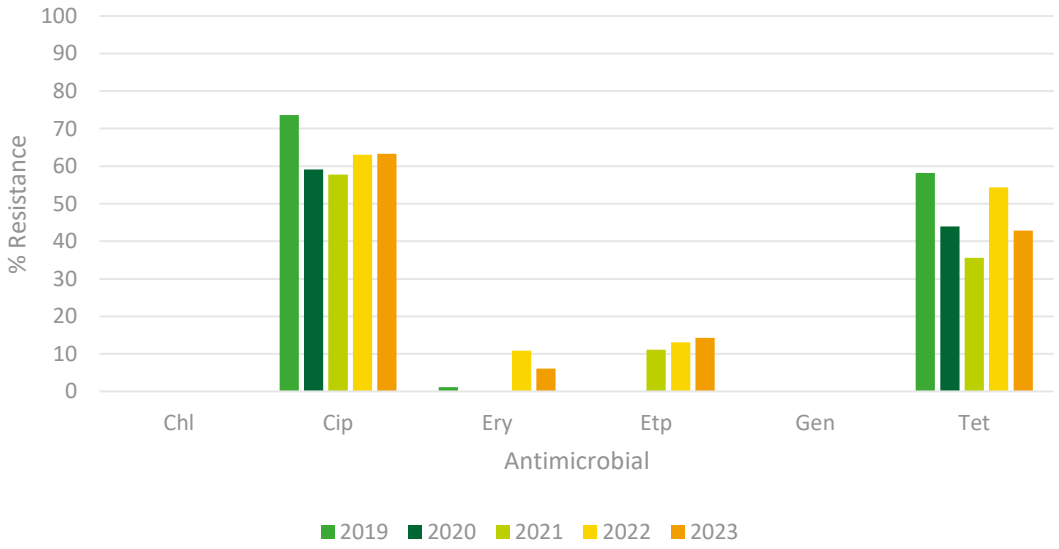

Figure 1. Trends in antimicrobial resistance rates in *C. jejuni* isolated from poultry meat (2019-2023).

In comparison with 2022, we see a decreased resistance to tetracycline (42.9%) and erythromycin (6.1%) in 2023 although these resistances remain higher than in 2021 (Figure 1).
The resistance level to ciprofloxacin (63.3%) was similar to that of 2022 and was also higher than in years 2020, 2021.
The resistance to ertapenem (14.3%) is particularly interesting as it keeps slowly increasing since its addition to the test panel in 2021 although the resistance level remains moderate.

The proportion of isolates susceptible to every antimicrobial tested is lower in 2022 and 2023 than in 2021 (Figure 2). This can be explained by the reappearance of resistance to erythromycin in 2022 after not being observed in 2021. Indeed, 5 isolates were resistant to erythromycin in 2022 and 3 in 2023.
Multi-drug resistance was lower in 2023 (10.2%) than in previous years with the exception of 2021 (which is also explained by the absence of erythromycin resistance detected that year).
To compare the numbers of resistances with the years before 2021, we need to take into account the change of antimicrobials included in the panel since then. Two antimicrobials were removed (nalidixic acid and streptomycin) but ertapenem and chloramphenicol were added. Resistance to ertapenem was found in *Campylobacter* spp. isolated from various matrices since 2021.

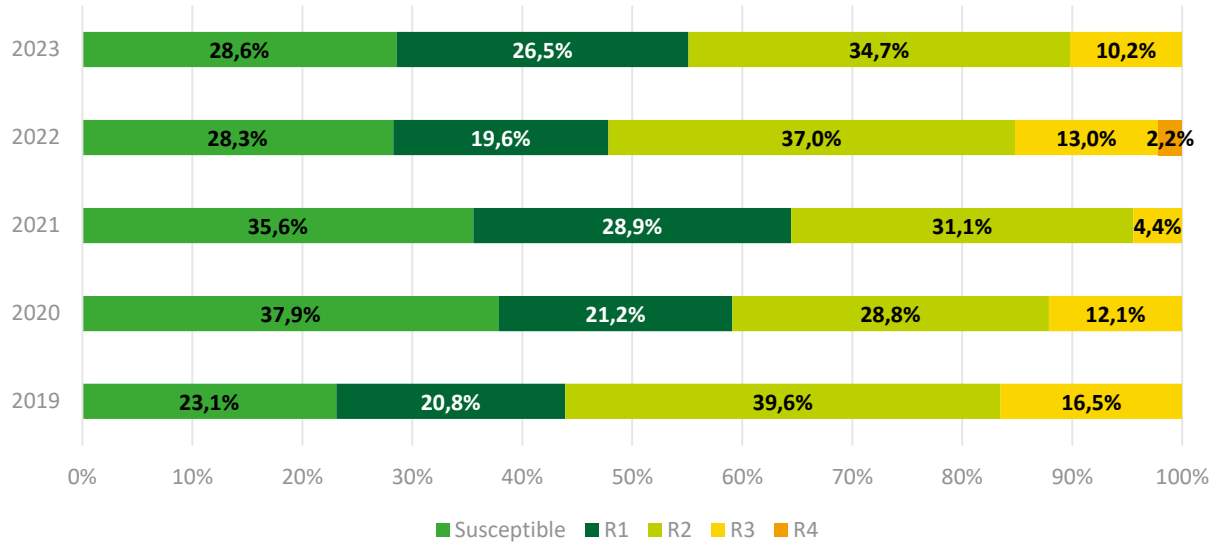

Figure 2. Percentages of susceptibility and resistance to one or more antimicrobial in *C. jejuni* (2019-2023)

In *Campylobacter coli* isolated from poultry meat in 2023, although resistance to ciprofloxacin decreased since 2022, it remained at an extremely high level (80%) which is higher than in 2021 (56.5%) (Figure 3). The extremely high resistance level detected in 2022 and 2023 compared to 2021 could be associated to closely related isolates dominating in the poultry sector presenting a common resistance profile. After a decrease in 2022, resistance to erythromycin, tetracycline and ertapenem increased in 2023, with resistances to ertapenem (74.3%) and tetracycline (77.1%) also higher than in 2021. Regarding the high resistance to ertapenem, it should be taken into account that there are no validated thresholds values for resistance recommended by EUCAST and that the Commission Implementing Decision does not specify the epidemiological cut-off to be used for ertapenem in *C. coli* and *C. jejuni*. An epidemiological threshold of 0,5 mg/L has been used in accordance to Société Française de Microbiogie in CA-SFM 2018 and CA-SFM 2019. The epidemiological cut-off recommended by EFSA is still under evaluation.

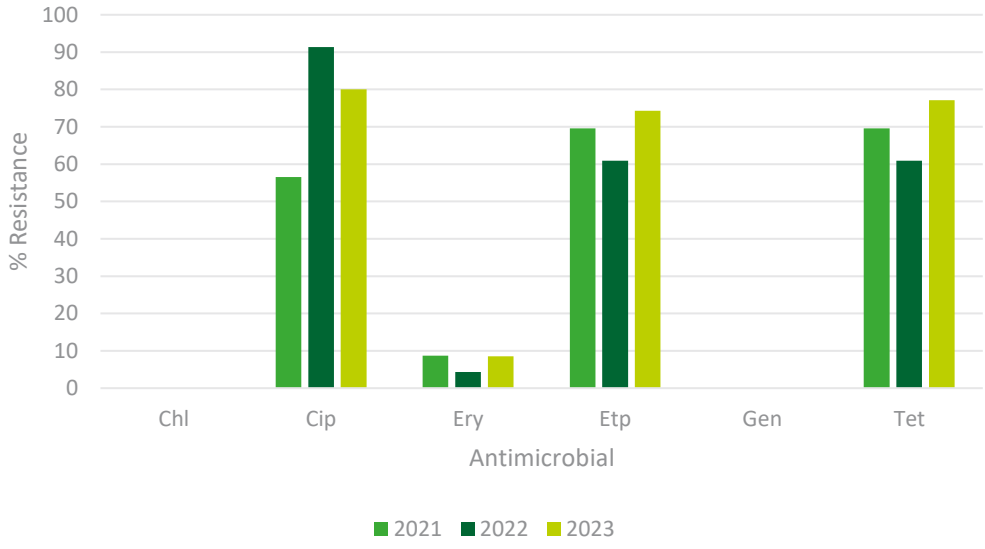

Figure 3. Trends in antimicrobial resistance rates in *C. coli* isolated from poultry meat (2021-2023)

The comparison of the resistance levels between *C. jejuni* and *C. coli* in 2023 (Figure 4) shows that, as expected, resistances are higher in *C. coli*. The difference is most noticeable in the case of resistance to ertapenem and tetracycline but also, to a lesser extent, ciprofloxacin. Resistance to chloramphenicol or gentamicin has not been detected in either *C. jejuni* or *C. coli* isolated from poultry meat samples.

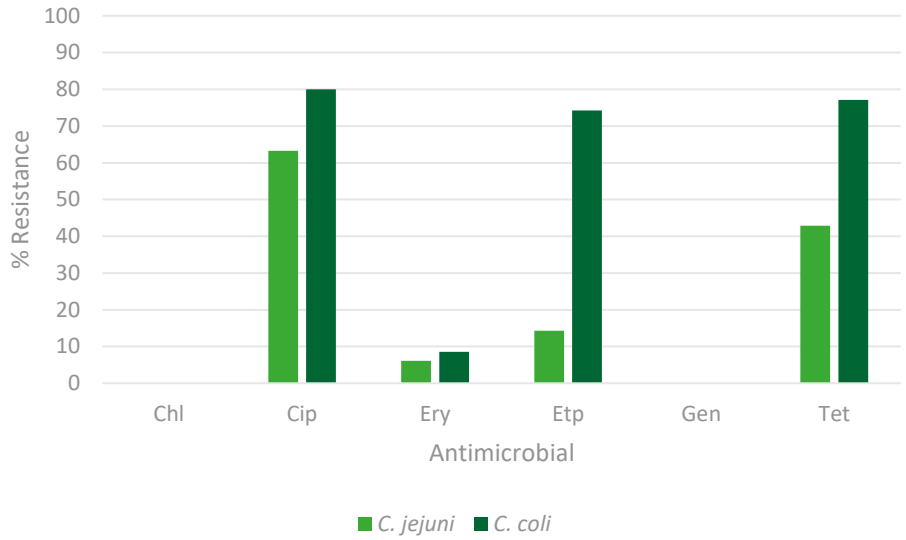

Figure 4. Levels of antimicrobial resistance in *C. jejuni* and *C. coli* isolated from poultry meat in 2023

3.1.2. Predicted antimicrobial resistance in *Salmonella* spp.

This section contains the analyses of the *Salmonella* food and feed programs.
Since 2023, *Salmonella* spp. retrieved from food matrices and feed were analysed by WGS as described in the section 2.1.6.

3.1.2.1. Whole genome sequencing of *Salmonella* spp. in Food

In 2023, 174 *Salmonella* spp. isolated from food matrices were analysed by WGS as described in the section 2.1.6. The number of isolates sequenced per food matrix are detailed in the table below (Table 12).

Table 12. Number of isolates sequenced per food matrix

Context	Description	Number of isolates
DIS 815	Meat preparations (pork, broiler or bovine)	6
DIS 819	Meat broiler (entire bird)	3
DIS 821	Poultry meat with or without skin	5
DIS 895	Vegetarian products	1
IEC 207	Sunflower seeds	2
PRI 004	Meat laying hen (entire bird)	2
PRI 028	Swabs from sheep carcasses	1
PRI 030	Swabs from bovine carcasses	5
PRI 031	Swabs from pig carcasses	46
PRI 034	Neck skin broiler	53
PRI 043	Organ's pork for consumption	6
TRA 100	Meat cuts (broiler, turkey, pork)	28
TRA 512	Seeds (coriander, pumpkin, sesame)	2
NA	Meat (unspecified)	11

The predicted resistance to antimicrobials on *Salmonella* spp. per food matrix is illustrated in the Figure 5 and results from whole genome sequencing are summarized in Table 13.

Due to the large variation in the number of isolates collected per matrix, data comparison is not pertinent. In addition, the antimicrobial resistance profile is often associated with specific serotypes, therefore description of the most relevant findings is discussed further.

Two out of the 174 isolates had a predicted resistance to 3rd generation cephalosporins, one isolated from sunflower seeds (IEC207) belonging to the serotype Kentucky (ST198). This isolate harbored a $bla_{CTX\text{-}M\text{-}14b}$ together with the following genes: *aac(3)-Id; aac(6')-Iaa, aadA7; aph(3'')-Ib, aph(3')-Ia; aph(6)-Id; sul1; tet(A)* which confer a multidrug resistant profile including sulfamethoxazole, tetracycline and gentamicin among other aminoglycosides. All *Salmonella* isolates harbored the resistance gene *aac(6')-Iaa* which codes for an aminoglycoside acetyltranferases that confers resistance to aminoglycosides. However, it has been reported that the presence of these genes does not correlate with resistance since they are often weakly expressed or not expressed.

The second isolate harboring a gene that confers resistance to extended spectrum β-lactams was isolated from cut poultry meat and belonged to the serotype Infantis (ST8662). The isolate harbored the following genes: $bla_{CTX\text{-}M\text{-}3}$, *aac(6')-Iaa; aadA1; aadA1; dfrA14; sul1 and tet(A)*.

One *Salmonella* Paratyphi B var. Java (ST28) harbored a plasmid mediated colistin resistance gene *mcr-9.1* and it was retrieved from broiler's neck skin (PRI034). The isolate carried the following genes as well, *aac(6')-Iaa; dfrA1; formA; lnu(F); aph(3')-Ia; sul1 and qacE* which predict a multidrug resistant phenotype including in addition to high priority critical important antimicrobials (represented by colistin) resistance to aminoglycosides, trimethoprim, fosfomycin, lincomycin, sulfamethoxazole and predicted resistance to biocidal quaternary ammonium compounds.

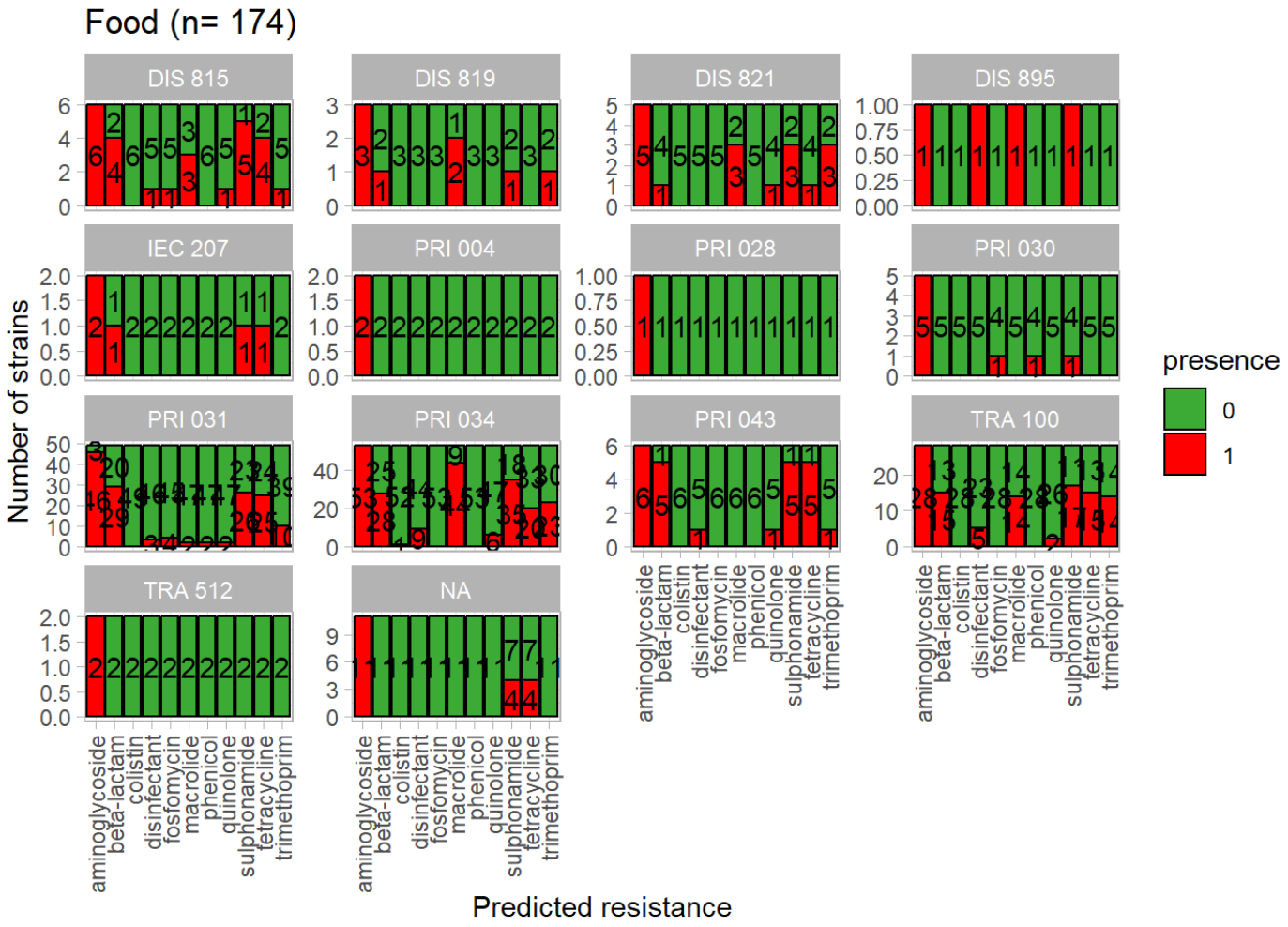

Figure 5. Figure 6. Predicted antimicrobial resistance phenotype per isolate on *Salmonella* spp. from food.

Table 13. Distribution of resistance genes to antimicrobials found in *Salmonella* spp. per food matrix.

gene	TRA 100	n=28	IEC 207	n=2	PRI 034	n=53	DIS 815	n=6	DIS 819	n=3	DIS 821	n=5	DIS 895	n=1	PRI 004	n=2	PRI 028	n=1	PRI 030	n=5	PRI 031	n=49	TRA 512	n=2
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
aac(3)-Id	0	0	1	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
aac(3)-IId	1	3,57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
aac(3)-VIa	0	0	0	0	1	1,89	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
aac(6')-Iaa	28	100	2	100	53	100	6	100	3	100	5	100	1	100	2	100	1	100	5	100	46	93,88	2	100
*aadA1**	6	21,43	0	0	8	15,09	1	16,67	0	0	1	20	0	0	0	0	0	0	1	20	0	0	0	0
aadA17	0	0	0	0	3	5,66	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
aadA1	0	0	0	0	1	1,89	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
aadA1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
aadA1	2	7,14	0	0	6	11,32	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	10,2	0	0
aadA15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2,04	0	0
aadA24	0	0	0	0	5	9,43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
aadA7	0	0	1	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ant(3'')-Ia	5	17,86	0	0	4	7,55	0	0	1	33,33	0	0	0	0	0	0	0	0	0	0	2	4,08	0	0
aph(3'')-Ib	2	7,14	1	50	0	0	2	33,33	0	0	0	0	0	0	0	0	0	0	0	0	21	42,86	0	0
aph(3')-Ia	4	14,29	0	0	15	28,3	2	33,33	1	33,33	2	40	1	100	0	0	0	0	0	0	0	0	0	0
aph(3')-Ia	3	10,71	1	50	4	7,55	1	16,67	1	33,33	1	20	0	0	0	0	0	0	0	0	0	0	0	0
aph(3')-Ia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2,04	0	0
aph(6)-Id	2	7,14	1	50	0	0	2	33,33	0	0	0	0	0	0	0	0	0	0	0	0	19	38,78	0	0

blaCARB-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	4,08	0	0
blaCTX-M-14b	0	0	1	50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
blaCTX-M-	1	3,57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
*blaTEM**	1	3,57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
blaTEM-1	13	46,43	0	0	28	52,83	4	66,67	1	33,33	1	20	0	0	0	0	0	0	0	0	27	55,1	0	0
catA1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	20	0	0	0	0
*dfrA1**	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	6,12	0	0
dfrA14	1	3,57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
dfrA14	5	17,86	0	0	6	11,32	1	16,67	1	33,33	0	0	0	0	0	0	0	0	0	0	1	2,04	0	0
dfrA17	1	3,57	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
dfrA1	7	25	0	0	17	32,08	0	0	0	0	3	60	0	0	0	0	0	0	0	0	5	10,2	0	0
dfrA5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2,04	0	0
erm(B)	1	3,57	0	0	2	3,77	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
floR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	4,08	0	0
formA	0	0	0	0	1	1,89	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
fosA7	0	0	0	0	0	0	1	16,67	0	0	0	0	0	0	0	0	0	0	1	20	4	8,16	0	0
lnu(F)	0	0	0	0	3	5,66	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
lnu(F)	3	10,71	0	0	12	22,64	1	16,67	1	33,33	2	40	1	100	0	0	0	0	0	0	0	0	0	0
lnu(G)	10	35,71	0	0	28	52,83	2	33,33	1	33,33	1	20	0	0	0	0	0	0	0	0	2	4,08	0	0
mcr-9_1	0	0	0	0	1	1,89	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
qacE	5	17,86	0	0	9	16,98	1	16,67	0	0	0	0	1	100	0	0	0	0	0	0	3	6,12	0	0
qacL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
qnrB19	1	3,57	0	0	6	11,32	0	0	0	0	1	20	0	0	0	0	0	0	0	0	2	4,08	0	0
qnrS1	1	3,57	0	0	0	0	1	16,67	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
sul1	14	50	1	50	35	66,04	3	50	1	33,33	3	60	1	100	0	0	0	0	1	20	4	8,16	0	0
sul2	1	3,57	0	0	3	5,66	1	16,67	0	0	0	0	0	0	0	0	0	0	0	0	2	4,08	0	0

sul2	2	7,14	0	0	0	0	2	33,33	0	0	0	0	0	0	0	0	0	0	0	0	20	40,82	0	0
sul2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2,04	0	0
tet(A)	12	42,86	1	50	17	32,08	2	33,33	0	0	1	20	0	0	0	0	0	0	0	0	3	6,12	0	0
tet(B)	2	7,14	0	0	1	1,89	2	33,33	0	0	0	0	0	0	0	0	0	0	0	0	21	42,86	0	0
tet(G)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	4,08	0	0
tet(M)	1	3,57	0	0	2	3,77	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

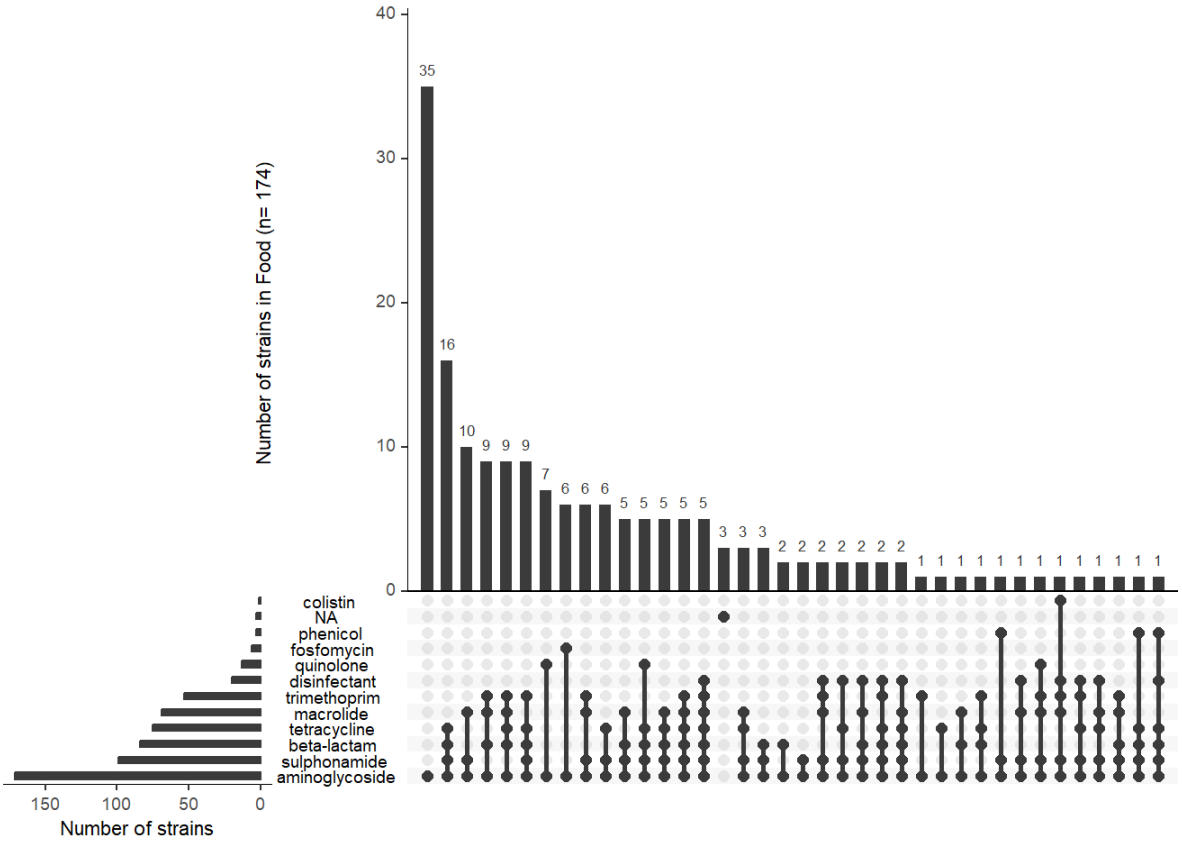

Figure 6. Predicted antimicrobial resistance phenotype per isolate on *Salmonella* spp. from food.

3.1.2.2. Whole genome sequencing of *Salmonella* spp. in Feed

In total 46 isolates were sequenced, of those 12 from animal protein feed (IEC 402), 18 from complete feed for dogs (TRA 082), 14 from land animal products and products derived thereof (TRA 085), and one from complementary feed for laying hens (PRI 041). Thirteen out of 18 (72%) *Salmonella* recovered from pet food were predicted to be resistant to at least three antimicrobial classes. Of those two *S.* Agona harbored genes conferring resistance to (fluoro)quinolones *qnrS*, streptomycin *aph(3')*, tetracycline (*tetA*) and fosfomycin (f*osA*). One *S*. Rissen, harbored the following resistant determinants: *aac(6')-Iaa; aadA2; bla$_{TEM-1B}$; catA2; dfrA12; mph(A), qnrB19, sul1; tet(A), qacE;* associated to resistance to ampicillin, chloramphenicol, trimethoprim, azithromycin, fluoroquinolones, sulfamethoxazole, tetracycline and quaternary ammonium compounds.

Isolates from the other feed matrices were not predicted to be resistant to any antimicrobial except occasionally to fosfomycin.

Figure 7 illustrates the predicted antimicrobial resistance profile per isolate.

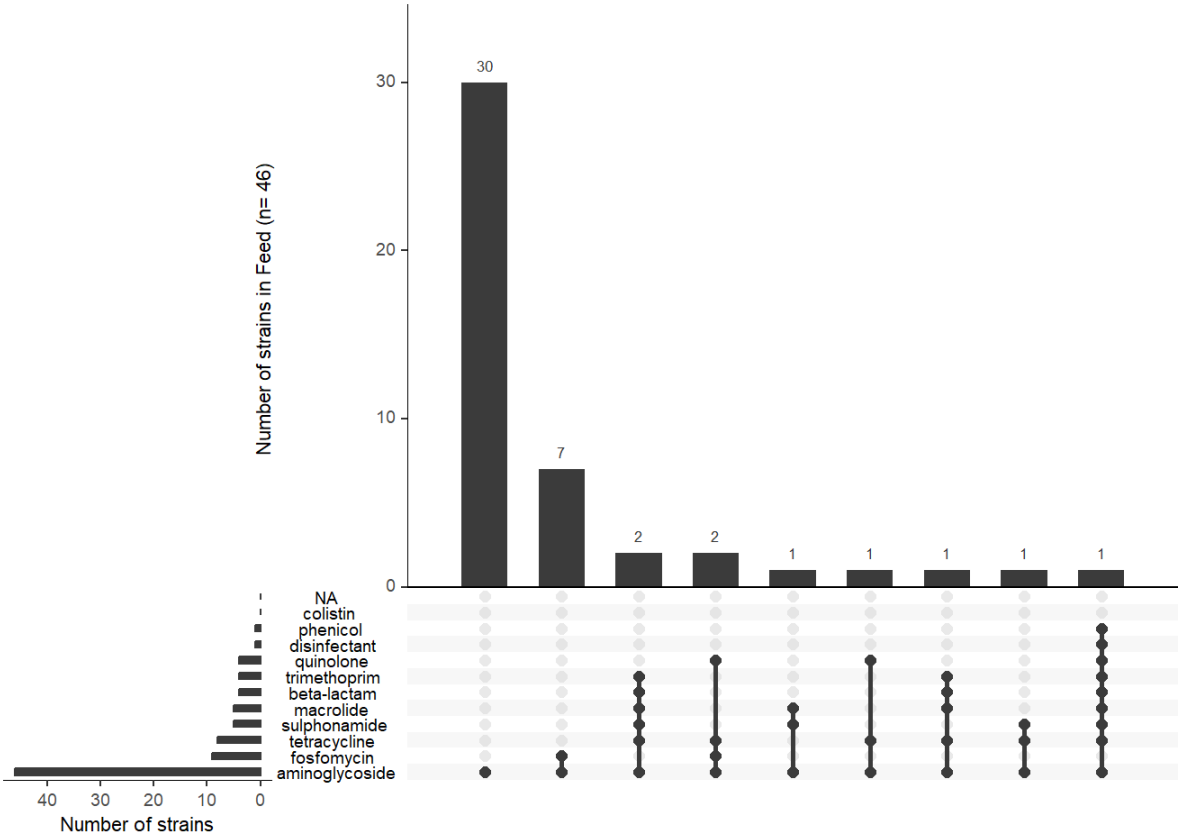

Figure 7. Predicted antimicrobial resistance phenotype per isolate on *Salmonella* spp. from feed matrices.

3.1.3. β-lactamases producing *E.coli*

3.1.3.1. Detection of ESBL, AmpC or carbapenemase producing *E.coli* in food matrices

The detection of β-lactamases producing *E.coli* was carried out according to the method described in the European Commission Implementing Decision 2020/1729. Samples of fresh meat from broiler (DIS 819, DIS 821), pig, bovine and turkey (DIS 809), and imported food IEC001 (poultry and beef meat) were tested for the detection of ESBL *E.coli*.

3.1.3.2. Specific monitoring of ESBL, AmpC or carbapenemase producing *E.coli* in broiler meat

In 2023, as part of the specific search for ESBL, AmpC or carbapenemases producing *E. coli* in broiler meat, a qualitative method (detection/non-detection in 25g) was carried out. The medium used is MacConkey combined with ready-to-use cefotaxime (CTX, 1 mg/L) (Biorad) for the detection of ESBL *E. coli* and/or AmpC and CarbaSmart medium (BioMérieux) for the detection of carbapenemases producing *E. coli*. Of 104 samples of fresh meat from broilers (DIS 819) and 208 samples of broiler meat preparations (DIS 821), 45 (43.27%) and 97 (46.63%) respectively were positive for the detection of ESBL *E.coli*. Hence, the prevalence of ESBL *E. coli* in broiler meat in 2023 is 45.51% compared to 58.33% in 2022. A gradual decrease of the prevalence of *E.coli* ESBL/AmpC has been noticed over the years. From 80% reported in 2016 to 45.51 % in 2023.

Imported fresh meat (IEC001) was tested for the presence of ESBL *E. coli*, from 15 samples from beef meat, none of them tested positive, however, all 3 imported poultry meat samples analysed, tested positive.

3.1.3.1. Specific monitoring of ESBL, AmpC or carbapenemase producing *E.coli* in bovine, pig and turkey meat

As part of the specific search for ESBL, AmpC or carbapenemase producing *E. coli* in bovine, pig and turkey meat (DIS 809), a qualitative method (detection/non-detection in 25g) was carried out. The medium used was MacConkey combined with ready-to-use cefotaxime (CTX, 1 mg/L) (Biorad) for the detection of ESBL and AmpC *E. coli* and CarbaSmart medium (BioMérieux) for the detection of carbapenemases.
313 samples of bovine meat, 312 samples of pig meat and 156 samples of turkey meat were tested for the detection of ESBL *E.coli* and 10 (3.19%), 6 (1.92%) and 25 (16.03%) samples were positive respectively.

The Figure 8 illustrates the prevalence of ESBL *E. coli* during the period 2017-2023 for all three categories of fresh meat: poultry, pork and beef.

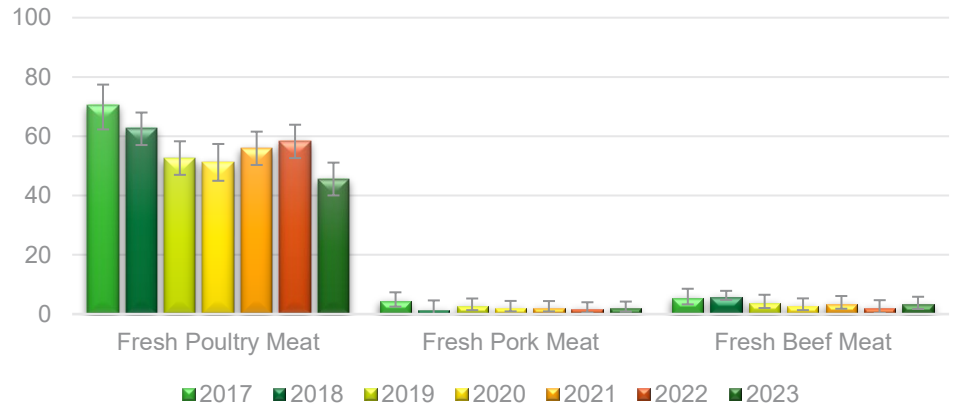

Figure 8. Prevalence of ESBL *E. coli* during 2017-2023 for all three categories of fresh meat, poultry, pork and beef.

WGS Data analysis:

Total number of isolates included in the data analysis are 6 from beef and pork meat respectively, 23 from turkey meat, 164 from poultry meat (DIS 819 and DIS 821) and 3 from imported poultry meat (IEC001).
Isolates which did not pass QC standards were eliminated from the analysis of the results.

The antimicrobial prediction profile of ESBL *E. coli* isolates based on resistance genes detected by WGS, is illustrated in the Figure 9. It has been accepted that accurate prediction of AMR phenotypes according to ResFinder application v. 4.4.2; ResFinder Database v. 2.2.1; PointFinder Database v. 4.0.1 can replace the need to do antimicrobial susceptibility testing.
Results illustrate the AMR profile of ESBL *E. coli* obtained from the different types of fresh meat: beef, pork, turkey and poultry.
All but 4 isolates have been confirmed as *E. coli* producing extended spectrum β-lactamases. Variation in the resistance profile is noted among the different types of fresh meat. None of the isolates were predicted to have resistance to meropenem. Predicted ciprofloxacin resistance varied among isolates from the different categories of fresh meat, being the highest detected in isolates retrieved from turkey meat, followed by poultry, and pork meat. The predicted prevalence of resistance to this antimicrobial is in line with resistance reported in previous years for broiler meat, 57.5% in 2023 vs 57.14% in 2022. In turkey meat predicted ciprofloxacin resistance accounted to 70% in 2023 compared to 75% in 2022.

None of the isolates collected from beef meat were resistant to that class of antimicrobials, however resistance to azithromycin was predicted for one and four isolates from beef and poultry meat, respectively. Predicted resistance to colistin was limited to one isolate from turkey meat.

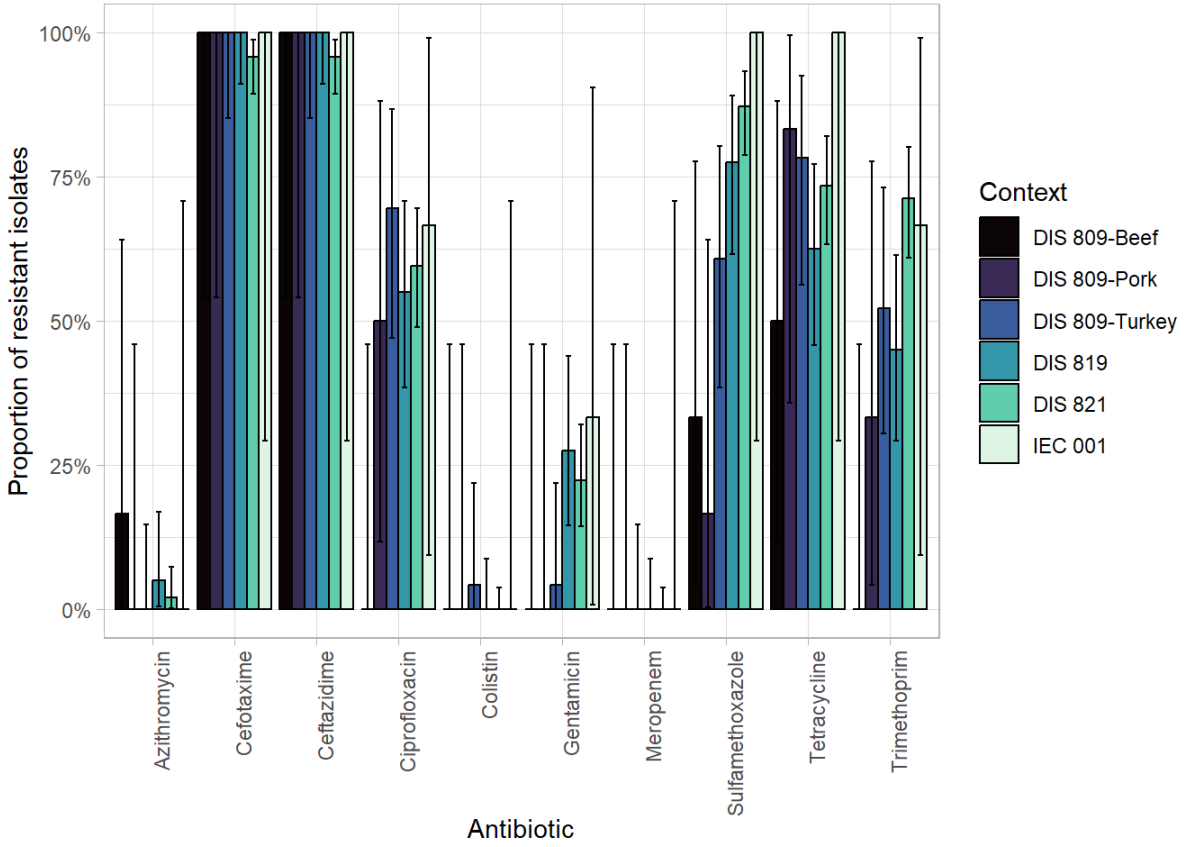

Figure 9. Predicted resistance to antimicrobials based on genotypic characterization by WGS.

The distribution of genes encoding for extended spectrum β-lactamase enzymes conferring resistance to 3rd generation cephalosporins, cefotaxime and/or ceftazidime are illustrated in the Figure 10 and Table 14. Prevalence of ESBL genes differed among the fresh meat sample groups. Isolates from poultry and turkey fresh meat harbored the highest number of resistance genes. In particular, the genes more frequently found conferring resistance to 3rd generation cephalosporins in isolates recovered from fresh poultry meat were *bla*$_{SHV-12}$ (47%, 63/134) followed by *bla* $_{CTX-M-55}$ (19.40%, 26/134) and *bla* $_{TEM-52}$ (11.94%, 16/134). Isolates from turkey fresh meat were found to carry the following ESBL genes, *bla*$_{SHV-12}$ (5/23, 21.7%), *bla* $_{CTX-M-27}$ (5/23, 21.7%), and *bla* $_{CTX-M-1}$ (4/23, 17.39%). Moreover 3 isolates harbored the gene *bla*$_{CMY-2}$ which confers resistance to cefoxitin and predict an AmpC phenotype.
Prevalence of ESBL genes among the isolates retrieved from pork meat were associated to 5 different determinants, *bla*$_{SHV-12}$ (1/6, 16%), *bla*$_{CTX-M-1}$ (1/6, 16%), *bla*$_{TEM-52}$ (1/6, 16%), *bla*$_{CMY-2}$ (1/6, 16%), *bla* $_{CTX-M-32}$ (1/6, 16%) and one isolate with no acquired resistance gene detected but with a point mutation in the ampC promoter (n.-42C>T). Four out of six isolates (66.67%) from beef fresh meat displayed similar mechanism of resistance, which is the most predominant one. This chromosomal mutation was observed as well in 4 isolates from poultry meat and 2 from turkey meat. The described mechanism confers resistance to cefoxitin and the predicted phenotype is AmpC.

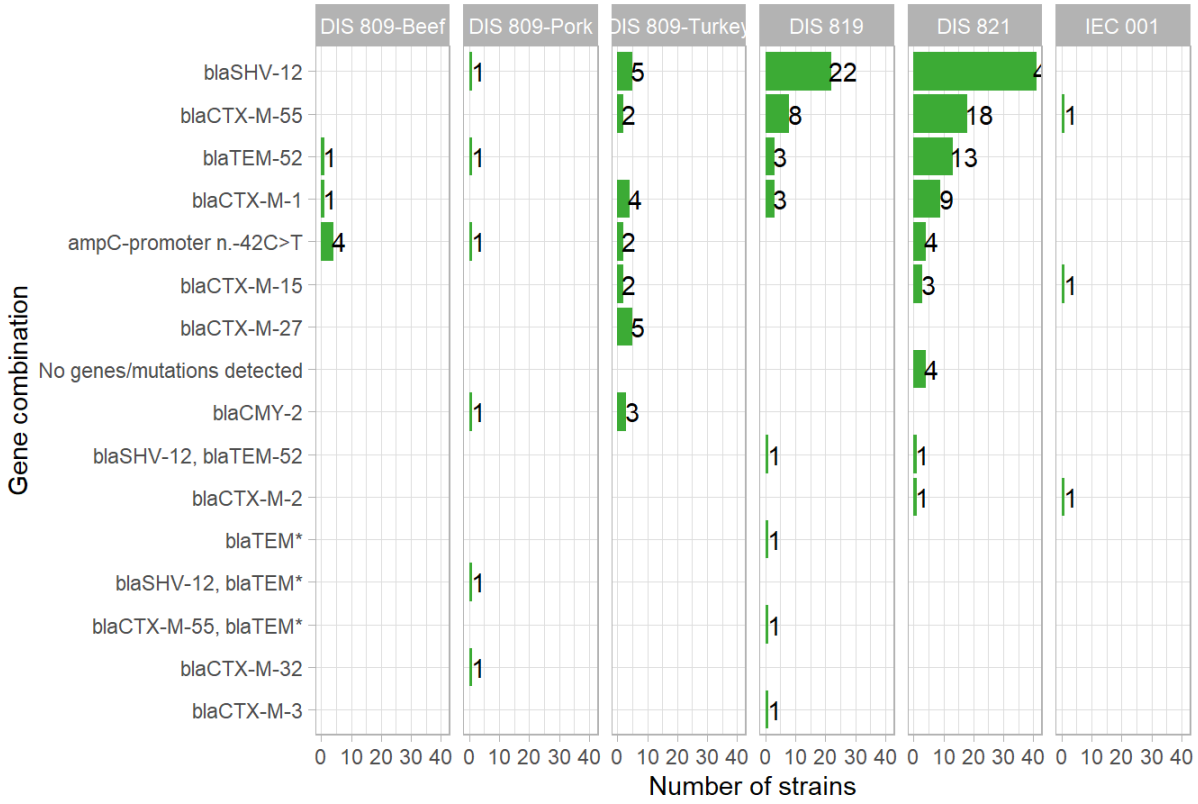

Figure 10. Distribution of genes/mutations encoding for extended spectrum β-lactamase enzymes conferring resistance to 3rd generation cephalosporins.

Table 14 Distribution of genes/mutations encoding production of extended spectrum β-lactamase enzymes in *E. coli* isolates from different fresh meat categories.

Genes/mutations	DIS 809-Beef n=6	%	DIS 809-Pork n=6	%	DIS 809-Turkey n=23	%	DIS 821	DIS 819	DIS 819/DIS 821 (n=134)	%	IEC 001 n=3	%	total
*bla*SHV-12	0	0,00	1	16,67	5	21,74	41	22	63	47,01	0	0,00	69
*bla*CTX-M-55	0	0,00	0	0,00	2	8,70	18	8	26	19,40	1	33,33	29
*bla*TEM-52	1	16,67	1	16,67	0	0,00	13	3	16	11,94	0	0,00	18
*bla*CTX-M-1	1	16,67	0	0,00	4	17,39	9	3	12	8,96	0	0,00	17
ampC-promoter n.-42C>T	4	66,67	1	16,67	2	8,70	4	0	4	2,99	0	0,00	11
*bla*CTX-M-15	0	0,00	0	0,00	2	8,70	3	0	3	2,24	1	33,33	6
*bla*CTX-M-27	0	0,00	0	0,00	5	21,74	0	0	0	0,00	0	0,00	5
*bla*CMY-2	0	0,00	1	16,67	3	13,04	0	0	0	0,00	0	0,00	4
*bla*SHV-12, *bla*TEM-52	0	0,00	0	0,00	0	0,00	1	1	2	1,49	0	0,00	2
*bla*CTX-M-2	0	0,00	0	0,00	0	0,00	1	0	1	0,75	1	33,33	2
*bla*TEM*	0	0,00	0	0,00	0	0,00	0	1	1	0,75	0	0,00	1
*bla*SHV-12, *bla*TEM*	0	0,00	1	16,67	0	0,00	0	0	0	0,00	0	0,00	1
*bla*CTX-M-55, blaTEM*	0	0,00	0	0,00	0	0,00	0	1	1	0,75	0	0,00	1
*bla*CTX-M-32	0	0,00	1	16,67	0	0,00	0	0	0	0,00	0	0,00	1
*bla*CTX-M-3	0	0,00	0	0,00	0	0,00	0	1	1	0,75	0	0,00	1
No genes/mutations detected	0	0,00	0	0,00	0	0,00	4	0	4	2,99	0	0,00	4

In conclusion, the following predicted phenotypes has been observed among the isolates from the different types of fresh meat:

Table 15. Proportion of isolates displaying an ESBL and AmpC phenotype based on genotyping characterization.

Predicted phenotype	DIS 809-Beef	DIS 809-Pork	DIS 809-Turkey	DIS 821/DIS819 Poultry	IEC 001
ESBL	16,67	66,67	78,26	94,02	100
AmpC	83,33	33,33	21,73	5,97	0

Those values are similar and in line with the values obtained in previous years by the antimicrobial susceptibility testing method.

ESBL *E. coli* resistant to ciprofloxacin

The distribution of plasmid mediated quinolone resistance genes (PMQR) and mutations in the quinolone resistance determining regions (QRDRs) are represented in the Figure 11 per each category of fresh meat.

Isolates from poultry meat (DIS 819-DIS821) accounted for the highest number of resistance genes, with 45 isolates (33.5%) harboring the PMQR *qnr*S gene, and 2.98% a *qnr*B. Regarding turkey meat, the distribution of the mentioned genes is 21% and 13% respectively. Regarding mutations in the QRDRs, we detected in *gyrA*, *parC* and *parE.* The most prevalent amino acid substitutions observed were S83L in *gyrA* in 32 (23.8%) isolates from poultry meat and 11 (47%) isolates from turkey meat.

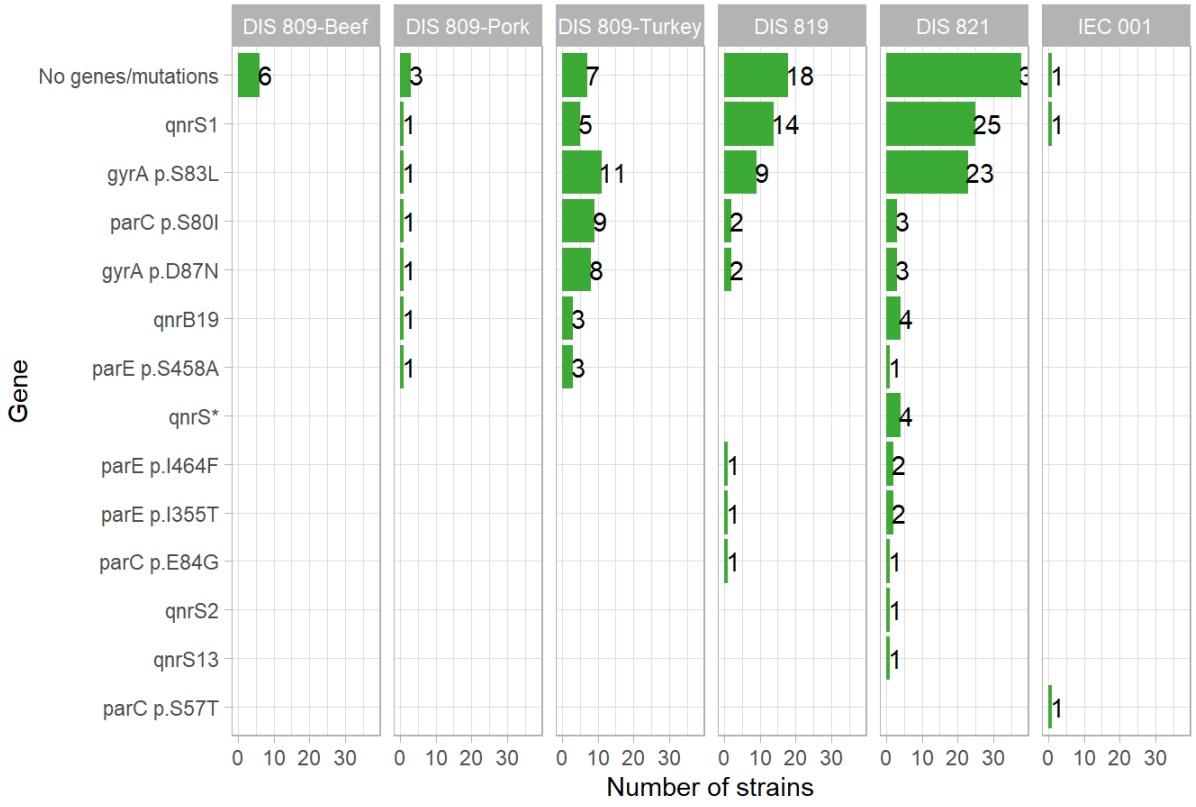

Figure 11. Distribution of resistant determinants inferring resistance to (fluoro)quinolones.

The combination of genes encoding for ESBL's and for (fluoro)quinolones resistance per each category of fresh meat are detailed in the Table 16.

Table 16. Combination of genes encoding for *ESBL's* and for (fluoro)quinolones resistance per each category of fresh meat.

Genes/mutations	DIS 809-Beef (n=6)	%	DIS 809-Pork (n=6)	%	DIS 809-Turkey (n=23)	%	DIS819/ DIS821 (n=134)	%	IEC 001 (n=3)	%
*bla*CTX-M-55, *qnrS1*	0	0	0	0	0	0	25,00	18,66	0	0
*bla*SHV-12, *gyrA* p.S83L	0	0	0	0	0	0	12,00	8,96	0	0
*bla*SHV-12, *qnrS1*	0	0	0	0	3,00	13,04	7,00	5,22	0	0
*bla*CTX-M-15, *qnrS1*	0	0	0	0	1,00	4,35	3,00	2,24	1,00	33,33
*bla*CTX-M-27, *gyrA* p.D87N, *gyrA* p.S83L, parC p.S80I	0	0	0	0	5,00	21,74	0	0	0	0
*bla*SHV-12, *qnrS**	0	0	0	0	0	0	4,00	2,99	0	0
ampC-promoter n.-42C>T, *gyrA* p.S83L	0	0	0	0	0	0	3,00	2,24	0	0
*bla*CMY-2, gyrA p.D87N, *gyrA* p.S83L, *parC* p.S80I, *parE* p.S458A	0	0	1,00	16,67	2,00	8,70	0	0	0	0
*bla*CTX-M-1, *gyrA* p.S83L	0	0	0	0	0	0	3,00	2,24	0	0
blaTEM-52, *gyrA* p.S83L, *parE* p.I464F	0	0	0	0	0	0	3,00	2,24	0	0
*bla*SHV-12, *qnrB19*	0	0	1,00	16,67	1,00	4,35	0,00	0,00	0	0
*bla*TEM-52, *gyrA* p.S83L	0	0	0	0	0	0	2,00	1,49	0	0
*bla*TEM-52, *qnrB19*	0	0	0	0	0	0	2,00	1,49	0	0
ampC-promoter n.-42C>T, *gyrA* p.D87N, *gyrA* p.S83L, *parC* p.S80I	0	0	0	0	0	0	1,00	0,75	0	0
*bla*CTX-M-1, *gyrA* p.D87N, *gyrA* p.S83L, *parC* p.E84G, *parC* p.S80I, *parE* p.I355T	0	0	0	0	0	0	1,00	0,75	0	0
*bla*CTX-M-1, *gyrA* p.S83L, *parC* p.S80I, *qnrB19*	0	0	0	0	1,00	4,35	0	0	0	0
*bla*CTX-M-1, *gyrA* p.S83L, *parE* p.I355T	0	0	0	0	0	0	1,00	0,75	0	0
*bla*CTX-M-1, *gyrA* p.S83L, *qnrB19*	0	0	0	0	1,00	4,35	0	0	0	0
*bla*CTX-M-1, *gyrA* p.S83L, *qnrS1*	0	0	0	0	1,00	4,35	0	0	0	0
*bla*CTX-M-1, *qnrS1*	0	0	0	0	0	0	1,00	0,75	0	0
*bla*CTX-M-1, *qnrS2*	0	0	0	0	0	0	1,00	0,75	0	0
*bla*CTX-M-15, *gyrA* p.D87N, *gyrA* p.S83L, *parC* p.S80I, *parE* p.S458A	0	0	0	0	1,00	4,35	0	0	0	0
*bla*CTX-M-2, *parC* p.S57T	0	0	0	0	0	0	0	0	1,00	33,33
*bla*CTX-M-55, *bla*TEM*, *qnrS1*	0	0	0	0	0	0	1,00	0,75	0	0
*bla*CTX-M-55, *qnrB19*, *qnrS1*	0	0	0	0	0	0	1,00	0,75	0	0
*bla*SHV-12, *bla*TEM*, *qnrS1*	0	0	1,00	16,67	0	0	0	0	0	0
*bla*SHV-12, *bla*TEM-52	0	0	0	0	0	0	1,00	0,75	0	0
*bla*SHV-12, *bla*TEM-52, *gyrA* p.S83L	0	0	0	0	0	0	1,00	0,75	0	0
*bla*SHV-12, *gyrA* p.D87N, *gyrA* p.S83L, *parC* p.E84G, *parC* p.S80I, *parE* p.I355T, *qnrS1*	0	0	0	0	0	0	1,00	0,75	0	0
*bla*SHV-12, *gyrA* p.D87N, *gyrA* p.S83L, *parC* p.S80I	0	0	0	0	0	0	1,00	0,75	0	0
*bla*SHV-12, qnrS13	0	0	0	0	0	0	1,00	0,75	0	0
*bla*TEM*, *gyrA* p.D87N, *gyrA* p.S83L, *parC* p.S80I	0	0	0	0	0	0	1,00	0,75	0	0

The most frequent combination of ESBL genes and determinants conferring resistance to (fluoro)quinolones found in turkey fresh meat was *bla*CTX-M-27 and mutation in the genes *gyrA* and *parC* (21.74%) followed by *bla*SHV-12, *qnrS* (13.04%). Isolates from poultry fresh meat, showed a diversity in

the combination of ESBL genes and fluro-(quinolones) genes. The most predominant one was *bla*$_{CTX-M-5}$, *qnrS* (18.66%), followed by *bla*$_{SHV-12}$, *gyrA* p.S83L (8.96%) and *bla*$_{SHV-12}$, *QnrS* (5.22%) .
The presence of (fluoro)quinolones genes in the ESBL *E. coli* isolates from pork revealed 3 different combinations, the first one *bla*$_{CMY-2}$, *gyrA* p.D87N, *gyrA* p.S83L, *parC* p.S80I, *parE* p.S458A, the second one *bla*$_{SHV-12}$, *QnrB19* and the third one *bla*$_{SHV-12}$, *bla*$_{TEM}$*, *qnrS1.* None of the ESBL isolates from beef meat carried a resistance gene or a mutation associated with resistance to (fluoro)quinolones.

ESBL *E. coli* resistant to gentamicin

The distribution of genes associated to resistance to gentamicin on ESBL *E. coli* isolates per each category of fresh meat are represented in the Figure 12.
The highest prevalence of resistance genes was found in poultry meat, with 32 (23.8%) isolates harboring one *aac(3)*-type gene as follows, *aac(3)-IId* 27 (20.15%), *aac(3)-IV* 3 (2.2%) and *aac(3)-Via* 2 (1.49%).
The presence of genes predicting resistance to gentamicin in ESBL *E. coli* isolates retrieved from pork and beef fresh meat was not detected. In turkey meat only one ESBL *E. coli* harbored an *aac(3)-IV* gene.

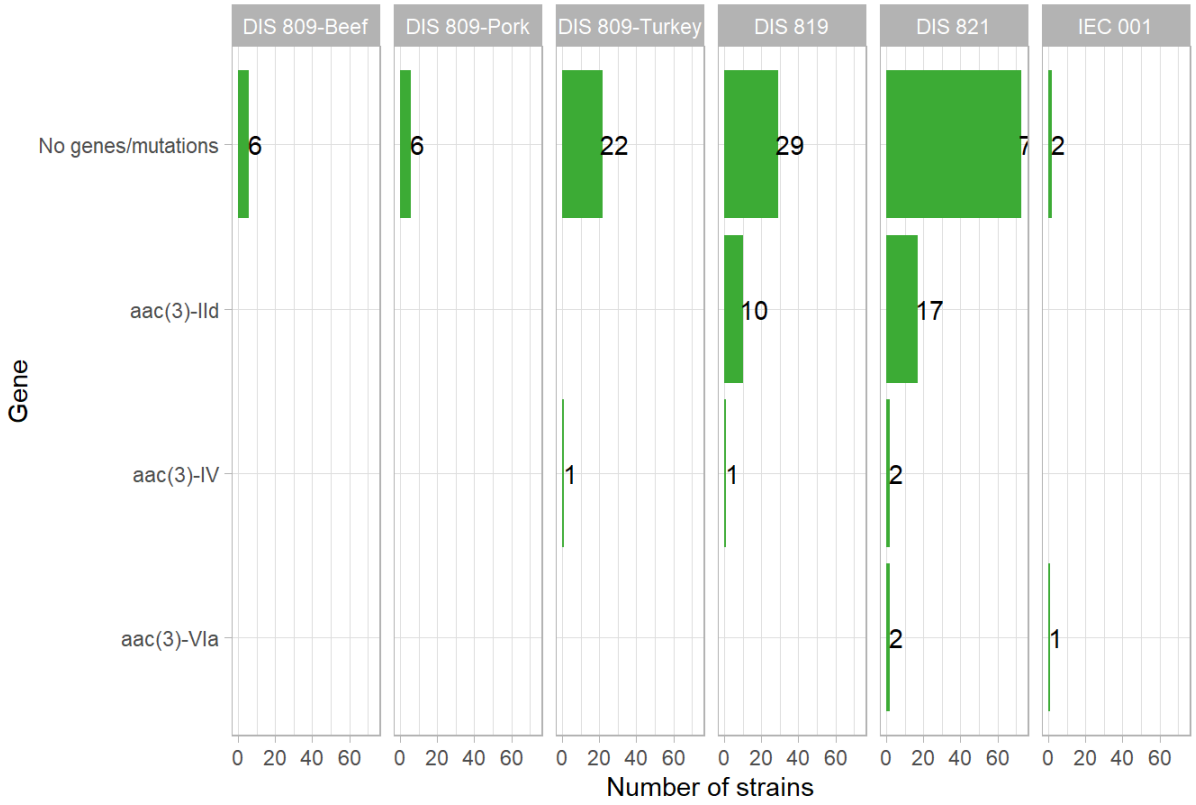

Figure 12. Distribution of resistant determinants inferring resistance to Gentamicin.

ESBL *E. coli* resistant to azithromycin

The distribution of genes associated to resistance to azithromycin on ESBL *E. coli* isolates are represented in the Figure 13 per each category of fresh meat.
Resistance to azithromycin is rare and genes associated were found in only 5 isolates, four from poultry meat and one from beef meat, all of them harbored the responsible gene *mph(A)*.

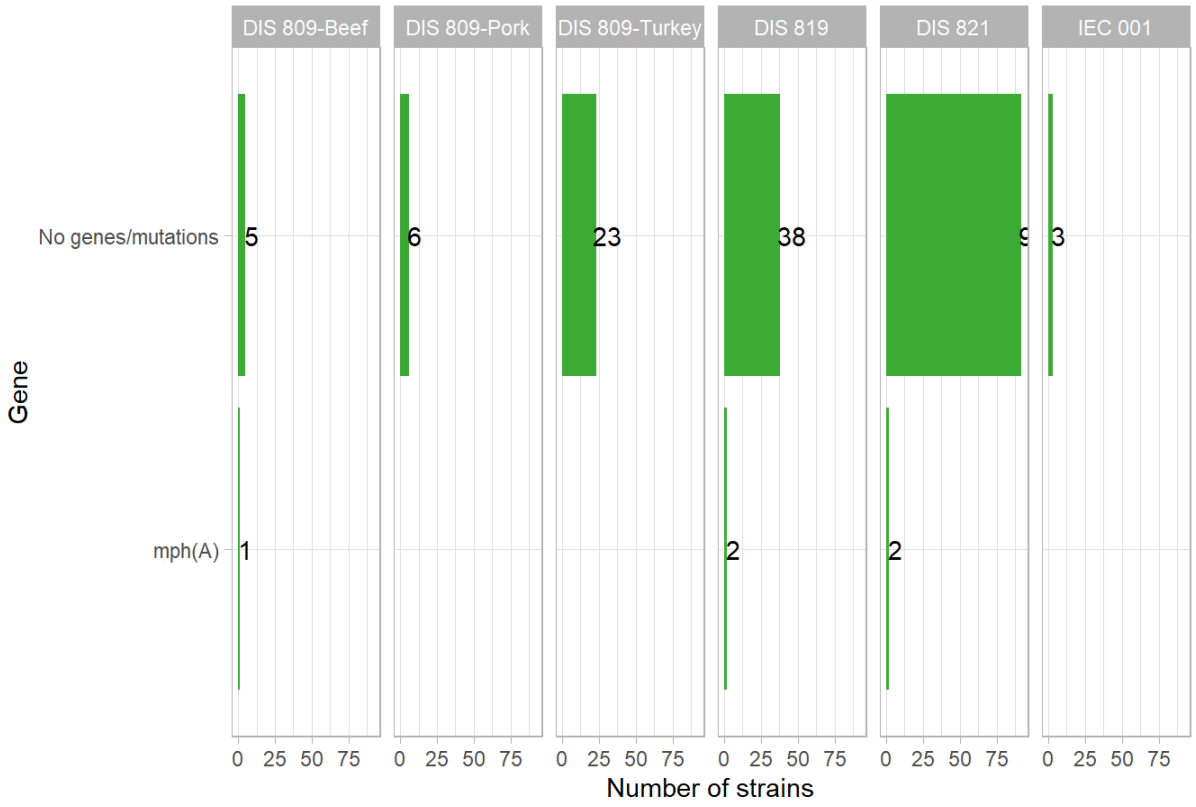

Figure 13. Distribution of resistant determinants inferring resistance to azithromycin.

3.2. ANTIMICROBIAL RESISTANCE MONITORING IN ZOONOTIC AND COMMENSAL BACTERIA ISOLATED FROM FOOD-PRODUCING ANIMALS (PRIMARY PRODUCTION)

In this section, the results of antimicrobial resistance monitoring in zoonotic and commensal bacteria isolated from populations of food-producing animals (primary production) are presented.

3.2.1. Monitoring of antimicrobial resistance in *Campylobacter* spp. isolated from broiler caecal content

In 2023, 294 samples of caeca from broilers were taken at the slaughterhouse for the detection of *Campylobacter* spp. and 142 were positive. Of those, 94 were identified as *C. jejuni* and 48 as *C. coli* by MALDI-TOF MS and 68 *C. jejuni* and 36 *C. coli* were subjected to antimicrobial susceptibility testing by broth microdilution method.

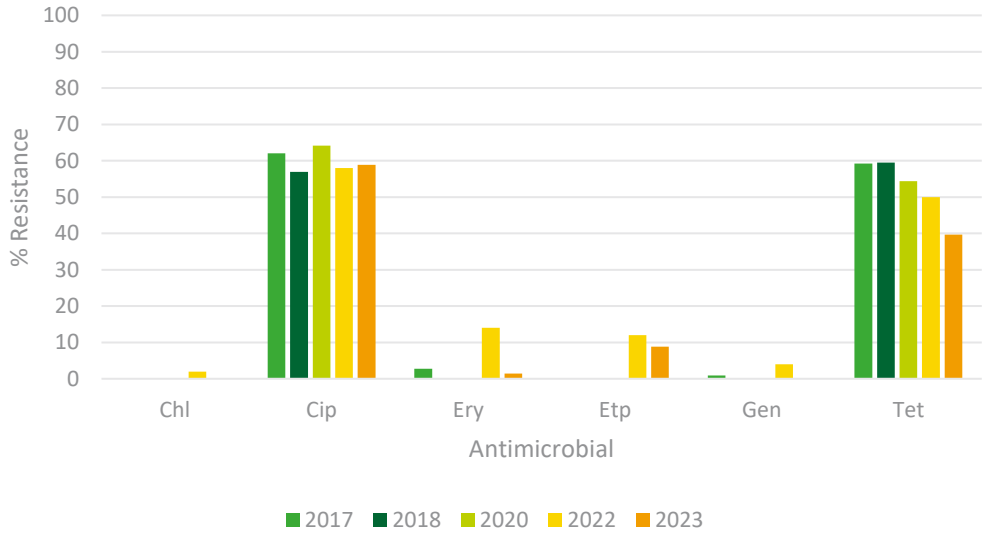

Figure 14. Trends in antimicrobial resistance rates in *C. jejuni* isolated from broiler caecal content

Unlike in 2022, resistances to chloramphenicol and gentamicin were not detected in *C. jejuni* isolated from broiler caecal content in 2023 (Figure 14). Resistance to erythromycin decreased almost tenfold from 14% to 1.5% and resistances to ertapenem (8.8%) and tetracycline (39.7%) also decreased in 2023. Resistance to ciprofloxacin (58.8%) remained at a very high level as in previous years.

In 2023, the monitoring was also conducted in 36 *Campylobacter coli* isolated from broiler caeca.

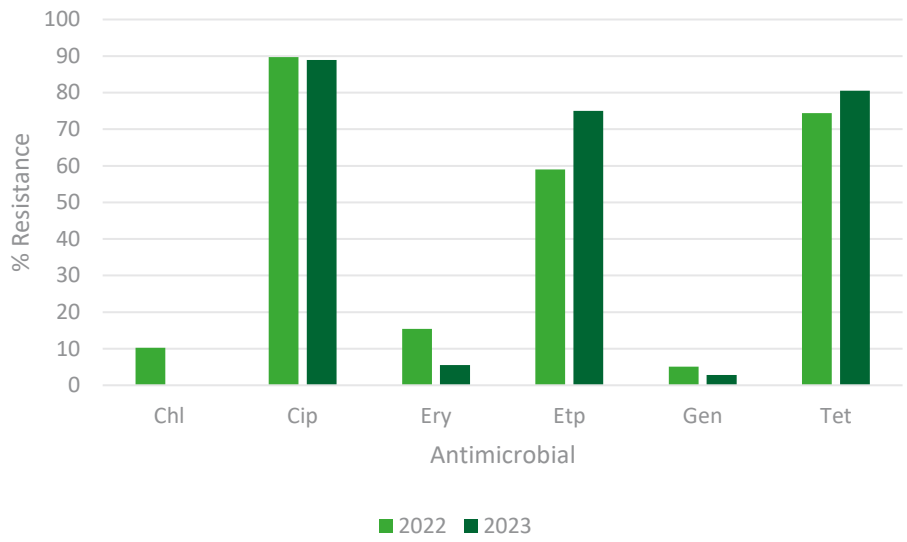

Figure 15. Trends in antimicrobial resistance rates in *C. coli* isolated from broiler caecal content

In comparison with 2022, there is an increased resistance to ertapenem (75%) and tetracycline (80.6%) in 2023 (Figure 15). However, resistance to ciprofloxacin (88.9%) remained extremely high but stable and resistances to erythromycin (5.6%) and gentamicin (2.8%) decreased. No resistance to chloramphenicol was detected in 2023 compared to a low resistance detected in 2022.

Figure 16 shows that higher levels of resistance were detected in isolates of *C. coli* than in *C. jejuni*. This is particularly true for resistance to ciprofloxacin and tetracycline and even more so for resistance to ertapenem. 63.9% of *C. coli* isolates were multidrug resistant and one (2.8%) was susceptible to all the antimicrobial tested whereas 5.88% of *C. jejuni* isolates were multidrug resistant and 36.8% were susceptible to all the antimicrobials tested.

The most common pattern in both *C. jejuni* and *C. coli* was resistance to both ciprofloxacin and tetracycline, observed in 36.8% of *C. jejuni* isolates and 77.8 % of *C. coli* isolates.

Regarding the resistance to ertapenem, it should be taken into account that there is no validation threshold for resistance recommended by EUCAST for *C. coli* and that the Commission Implementing Decision does not specify the epidemiological cut-off to be used for ertapenem in *C. coli* and *C. jejuni*. An epidemiological threshold of 0.5 mg/L has been used in accordance to Société Française de Microbiogie in CA-SFM 2018 and CA-SFM 2019. EUCAST also recently reduced the ECOFF for the determination of ertapenem in *C. jejuni* to 0.125 mg/L but the epidemiological cut-off recommended by EFSA and EURL is still in analysis so the previous value of 0.5 mg/L is still applicable in this report.

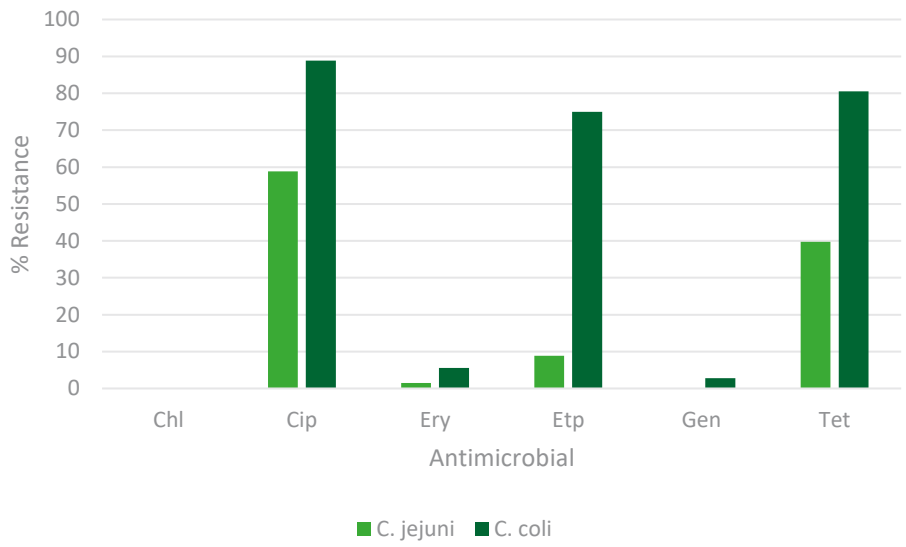

Figure 16. Comparison of resistance levels in *C. jejuni* and in *C. coli* isolated from broiler caecal content in 2023

3.2.2. Monitoring of antimicrobial resistance in *Campylobacter coli* isolated from pig caecal content

In 2023, 298 samples of pig caeca taken at the slaughterhouse were tested for the detection of *Campylobacter coli* and 198 were positive. The minimal inhibitory concentration (MIC) was determined for 174 isolates (Figure 17).

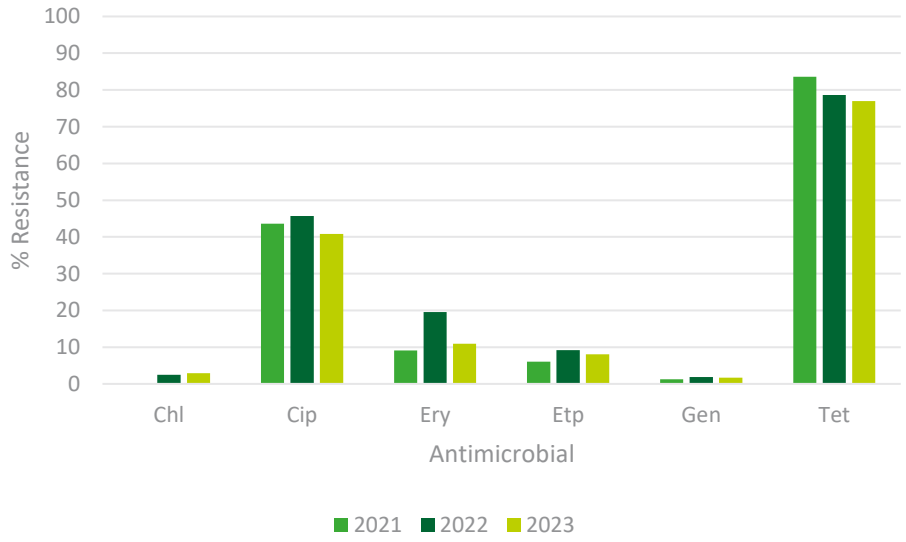

Figure 17. Trends in resistance rates in *C. coli* isolated from pig caecal content (2021-2023)

Figure 17 shows that overall, resistances were stable or slightly decreasing in 2023 in comparison with 2022. Resistance to tetracycline (77%) remained extremely high but is in a decreasing trend since 2021. Resistance to ciprofloxacin (40.8%) was high and resistance to erythromycin was moderate (10.9%). The most common core pattern of resistance included ciprofloxacin and tetracycline, 62 out of 174 (22.6%) isolates were resistant to both antimicrobials. Combined resistance to critically important antimicrobials ciprofloxacin and erythromycin accounted for 5.7% (10 out of 174 isolates). Moreover 9 out of these 10 isolates also showed resistance to tetracycline. 8% of isolates were multidrug resistant and 16.7% were susceptible to all antimicrobials tested.

3.2.3. Monitoring of antimicrobial resistance in *Campylobacter* spp. isolated from bovine caecal content

300 samples of bovine caecal content were taken at the slaughterhouse in 2023 for the detection of *Campylobacter* spp. isolates and 287 were positive. Of those, 168 *C. jejuni* and 88 *C. coli* were subjected to antimicrobial susceptibility testing.

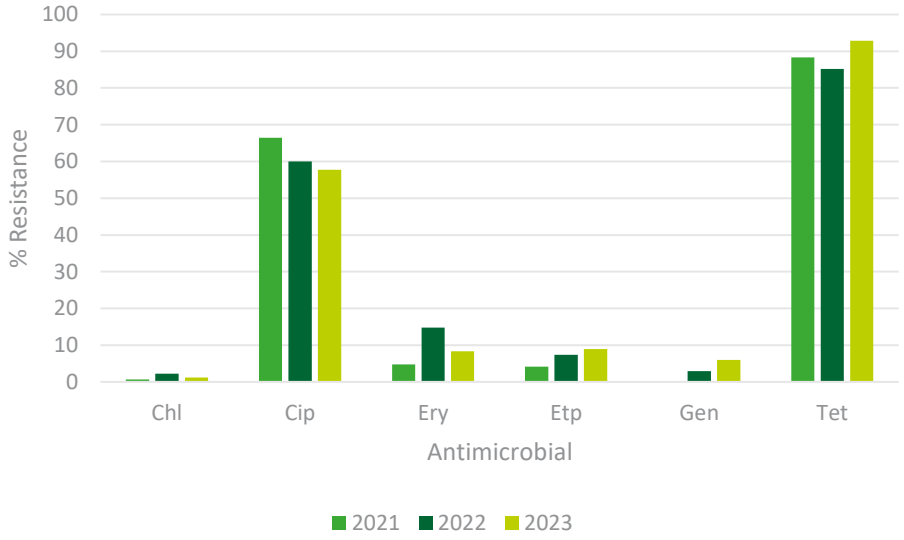

Figure 18. Trends in resistance rates in *C. jejuni* isolated from bovine caecal content (2021-2023)

In *Campylobacter jejuni*, resistance to tetracycline was higher in 2023 than in 2022 and 2021 with more than 90% of the isolates showing resistance. Resistance to ertapenem (8.9%), while remaining at a low level, also kept increasing since 2021 and resistance to gentamicin (6%), first detected in 2022 was also higher in 2023. However, resistance to ciprofloxacin (57.7%) is on a decreasing trend since 2021 but remains at a very high level. Resistances to chloramphenicol and erythromycin remained low and also decreased in comparison with 2022. (Figure 18).
The most common core pattern of resistance included ciprofloxacin and tetracycline, 92 out of 168 (54.8%) isolates were resistant to both antimicrobials. Combined resistance to critically important antimicrobials ciprofloxacin and erythromycin accounted for 6.5% (11 out of 168 isolates). Moreover these 11 isolates were also all resistant to tetracycline.

In 2023, 23 isolates of *C. jejuni* were multidrug resistant (13.7%) and 6 were susceptible to all antimicrobials tested (3.6%).

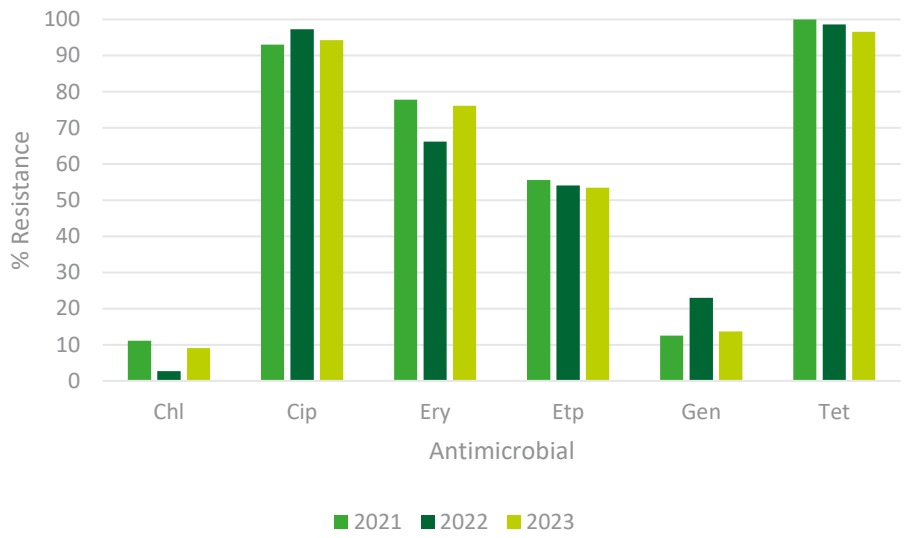

Figure 19. Trends in resistance rates in *C. coli* isolated from bovine caecal content (2021-2023)

Figure 19 shows that in *Campylobacter coli* resistance to ertapenem (53.4%) and tetracycline (96.6%) are slowly decreasing since 2021. Ciprofloxacin resistance remained extremely high but also decreased in comparison with 2022. However, after a decrease in 2022, erythromycin (76.1%) and chloramphenicol (9.1%) resistances increased in 2023 and the resistance levels are more similar to those found in 2021. Conversely, resistance to gentamicin (13.6%), decreased to a similar level as in 2021 after an increase in 2022. As expected, the level of multidrug resistance was much higher in *C. coli* than in *C. jejuni* with 90.9% of *C. coli* isolates resistant to 3 or more classes of antibiotics. Moreover, no *C. coli* isolate was susceptible to all antimicrobials tested.

82 out of 88 isolates (90.9%) were resistant to ciprofloxacin and tetracycline. Combined resistance to critically important antimicrobials ciprofloxacin and erythromycin accounted for 75% of the isolates (66 out of 88). Moreover, this pattern was accompanied by resistance to tetracycline as well in 65 of the 66 isolates (CipEryTet).

The difference in the levels of resistance of *C. coli* and *C. jejuni* is represented in Figure 20.

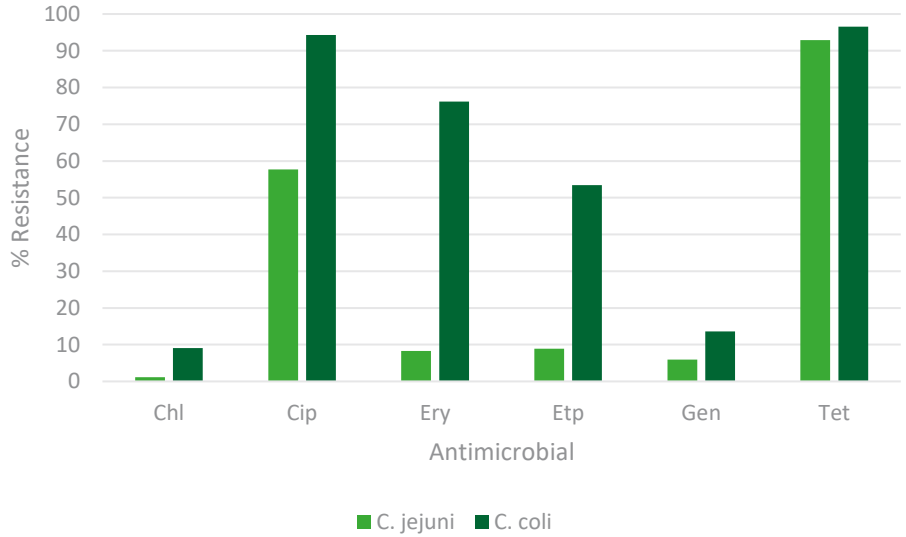

Figure 20. Comparison of resistance rates in *C. jejuni* and in *C. coli* isolated from bovine caecal content in 2022

Results show that, as expected, resistance is much in *C. coli* than in *C. jejuni.* This is especially true for resistances to ciprofloxacin, erythromycin and ertapenem.

3.2.4. Monitoring of antimicrobial resistance in *Salmonella* spp. isolated from pig caecal content

As part of the monitoring of *Salmonella* spp in samples of pig caeca collected at the slaughterhouse, 298 samples were collected and 47 were positive. Of those, 46 isolates of *Salmonella* spp. were subjected to antimicrobial susceptibility testing.
Figure 21 shows the percentage of *Salmonella* serotypes identified in pig caeca. The most prevalent serotype is monophasic Typhimurium (30%), followed by Typhimurium (26%) and Derby (20%).

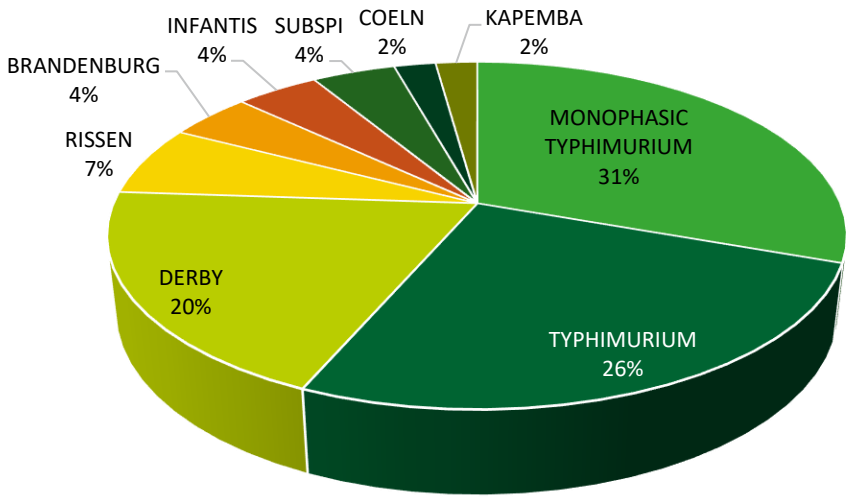

Figure 21. Percentage of serotypes identified for *Salmonella* isolated from pig caecal content

Figure 22 shows the resistance rate of all *Salmonella* spp. isolated from pig caeca and tested for antimicrobial susceptibility. Resistance to amikacin was detected for the first time in 2023 in an isolate of *Salmonella* (Subspi : I 4:d-) isolated from pig caecal content. For all the other antimicrobials, except trimethoprim, a decreased resistance was detected in 2023 in comparison with 2022. Resistance to (fluoro)quinolones also decreased to lower levels than those detected in 2021. As in 2022, no resistance to azithromycin, 3rd generation cephalosporins or meropenem was detected.
43.5% of the isolates were multidrug resistant and 41.3% were susceptible to all antimicrobial tested. Considering the serotype Typhimurium and its monophasic variant, 76.9% of the isolates were resistant to ampicillin, followed by 65.4% to sulfamethoxazole and 61.5% to tetracycline. None of them were resistant to ciprofloxacin. Resistance to ciprofloxacin was only observed in 1 isolate, belonging to serotype Derby. Among the 3 most prevalent serotypes, the Monophasic Typhimurium serotype contained the most multidrug resistant isolates while the Derby serotype contained the most fully susceptible isolates.

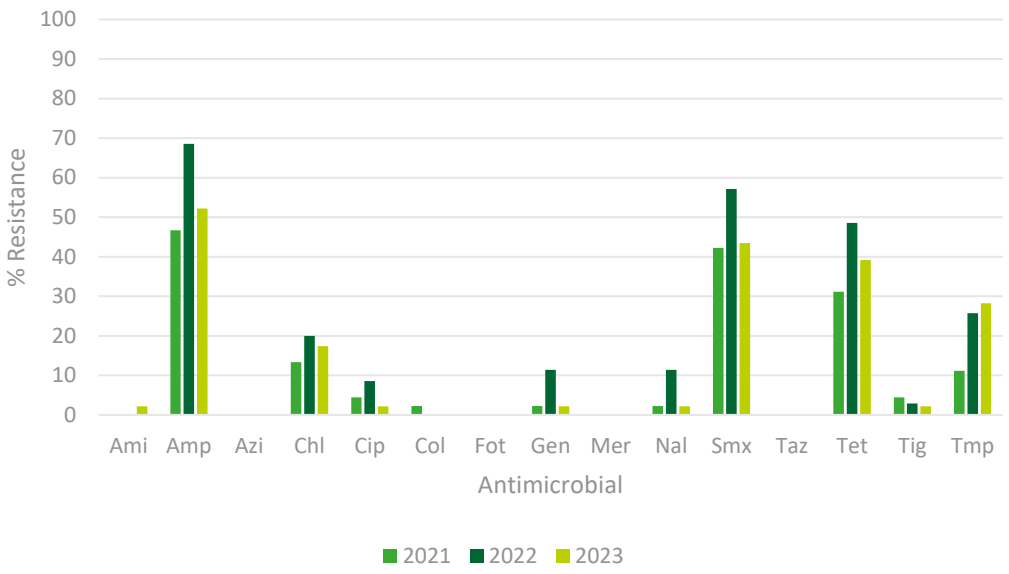

Figure 22. Trends in resistance rates in *Salmonella* spp. isolated from pig caecal content (2021-2023)

3.2.5. Monitoring of antimicrobial resistance in *Salmonella* spp. isolated from bovine caecal content

Out of 300 samples of bovine caecal content taken at the slaughterhouse tested for the presence of *Salmonella* spp. 3 were positive and the isolates were subjected to antimicrobial susceptibility testing. 2 of the 3 isolates belonged to the Typhimurium serotype and one to the Monophasic Typhimurium variant. The isolates of *S*. Typhimurium were both resistant to tetracycline and sulfamethoxazole only and the *S*. Monophasic Typhimurium was resistant to ampicillin, chloramphenicol, sulfamethoxazole and trimethoprim.

WGS of *Salmonella* spp.

All the *Salmonella* spp. isolates recovered from the food producing animals, fattening pigs (PRI 035), bovines (PRI 036) sampled at slaughterhouse and from the official control on laying hens (PRI 518) and broilers (PRI 519) sampled at farm were sequenced for detection of AMR genes and predict phenotypic resistance.

In total 72 isolates were included in the data analysis, 43 from caecal content of fattening pigs, 3 from bovines, 12 from laying hens and 8 from broilers.
The Figure 23 illustrates the predicted phenotypic resistance to ten antimicrobial families and in addition the biocides.
All *Salmonella* isolates harbored the resistance gene *aac(6')-Iaa* which codes for an aminoglycoside acetyltranferases which confers resistance to aminoglycosides. However, it has been reported that the presence of these genes does not correlate with resistance since they are often weakly expressed or not expressed. The presence of other associated aminoglycosides genes were found in *Salmonella* from fattening pigs, *aph(3'')*, aph(6)-Id, in addition to *fosA*, *sul2*, *tet(B)*, which confer resistance to fosfomycine, sulfamides and tetracycline.
None of the isolates were predicted to be resistant to highest priority critically important antimicrobials particularly to 3rd generation cephalosporins or to colistin.
Resistance to ciprofloxacin was predicted in one isolate from broilers at farm, by the detection of the gene *qnr*B.

All genes present in the Salmonella isolates are listed in the Table 17 per food producing animal category.

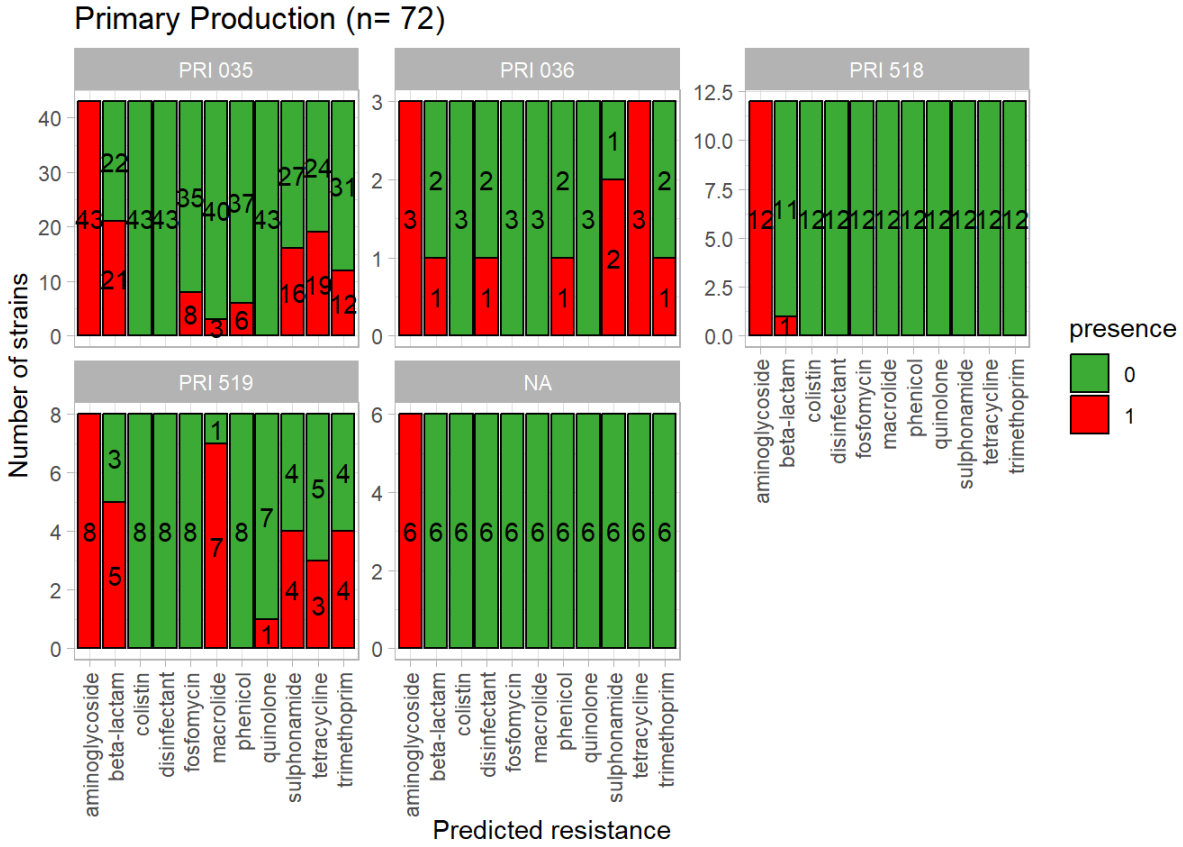

Figure 23. Predicted resistance to antimicrobials based on genotypic characterization by WGS.

Table 17. Distribution of resistant genes found in *Salmonella* spp. per category of food producing animals

gene	Fattening pigs PRI 035	%	Bovines PRI 036	%	Laying hens PRI 518	%	Broilers PRI 519	%
aac(6')-Iaa_1	43	100,00	3	100,00	12	100	8	100,00
aadA1_3	4	9,30	1	33,33	0	0	0	0
aadA1_4	5	11,63	0	0	0	0	2	25,00
aadA1_5	2	4,65	0	0	0	0	0	0
aadA2_2	4	9,30	1	33,33	0	0	0	0
aadA2b_1	2	4,65	0	0	0	0	1	12,50
ant(3'')-Ia_1	2	4,65	0	0	0	0	1	12,50
aph(3'')-Ib_5	16	37,21	3	100,00	0	0	0	0
aph(3')-Ia_1	0	0	0	0	0	0	1	12,50
aph(3')-Ia_7	0	0	0	0	0	0	2	25,00
aph(3')-Ia_9	1	2,33	0	0	0	0	0	0
aph(6)-Id_1	13	30,23	3	100,00	0	0	0	0
bla_{CARB-2}	1	2,33	0	0	0	0	0	0
bla_{TEM-1}	20	46,51	1	33,33	1	8,33	5	62,50
cmlA1_1	5	11,63	1	33,33	0	0	0	0
*dfrA1**	3	6,98	0	0	0	0	1	12,50
dfrA12_8	4	9,30	1	33,33	0	0	0	0

dfrA14_4	0	0	0	0	0	0	1	12,50
dfrA1_8	5	11,63	0	0	0	0	2	25,00
erm(B)_1	0	0	0	0	0	0	1	12,50
floR_2	1	2,33	1	33,33	0	0	0	0
fosA7_1	8	18,60	0	0	0	0	0	0
lnu(F)_1	0	0	0	0	0	0	1	12,50
lnu(F)_3	0	0	0	0	0	0	1	12,50
lnu(G)_1	2	4,65	0	0	0	0	4	50,00
mph(B)_1	1	2,33	0	0	0	0	0	0
qacL_1	0	0	1	33,33	0	0	0	0
qnrB19_1	0	0	0	0	0	0	1	12,50
sul1_2	4	9,30	0	0	0	0	3	37,50
sul2_2	1	2,33	2	66,67	0	0	1	12,50
sul2_3	13	30,23	0	0	0	0	0	0
sul3_2	1	2,33	0	0	0	0	0	0
tet(A)_4	2	4,65	2	66,67	0	0	2	25,00
tet(B)_2	16	37,21	0	0	0	0	0	0
tet(G)_2	1	2,33	0	0	0	0	0	0
tet(M)_4	0	0	0	0	0	0	1	12,50
tet(M)_8	4	9,30	1	33,33	0	0	0	0

3.2.6. Monitoring of commensal and ESBL/AmpC/carbapenemase producing *E.coli*

Table 18 shows the number of commensal indicator *E. coli* isolated from food-producing animals and tested for antimicrobial susceptibility. The results of the susceptibility testing are shown below.

Table 18. Total number of commensal *E.coli* isolated from food-producing animals and subjected to one or both panels of antimicrobials in 2023.

Programme	Technical sheet	Reported AST
Broilers - caeca	PRI 019 (broilers)	
AST 1st panel		171
AST 2nd panel		5
Breeding hens - faeces	PRI 515	
AST 1st panel		165
AST 2nd panel		1
Laying hens - faeces	PRI 515	
AST 1st panel		176
AST 2nd panel		1
Bovines (slaughterhouse) - caeca	PRI 036	
AST 1st panel		171
AST 2nd panel		0
Bovines (farm) - faeces	PRI 515	
AST 1st panel		171
AST 2nd panel		3
Fattening pigs - caeca	PRI 035	
AST 1st panel		176

AST 2nd panel	0

3.2.7. Monitoring of antimicrobial resistance in indicator commensal *E. coli* isolated from caecal content of poultry, pigs and bovines

In 2023, samples of the caecal content of broilers (PRI 019), of pigs (PRI 035) and of bovines (PRI 036) were collected at the slaughterhouse and analysed in accordance with the Commission Implementing Decision 2020/1729.
Faecal samples of bovines less than 7 months old as well as from breeding and laying hens were also collected at farm level. Isolation of commensal *E. coli* was done at the FASFC laboratories and the isolates were sent to the NRL AMR (Sciensano) for antimicrobial susceptibility testing.

Table 19. Samples tested for the detection of commensal *E.coli* in 2023

Technical sheet	Description	Sampling Location	Samples Tested	*Samples positive for E. coli*
PRI 019	Broilers	Slaughterhouse	178	178
PRI 035	Fattening pigs	Slaughterhouse	180	180
PRI 036	Bovines	Slaughterhouse	180	180
PRI 515	Bovines	Farm	177	177
PRI 515	Breeding hens	Farm	169	165
PRI 515	Laying hens	Farm	180	176

3.2.7.1. Monitoring of indicator commensal *E.coli* in broilers caecal content.

In 2023, 178 samples of broiler caeca were tested for the detection of *E.coli* and all were positive. Of those, 171 *E.coli* isolates were tested for antimicrobial susceptibility. The results of the minimal inhibitory concentration analysis are represented in Figure 24.

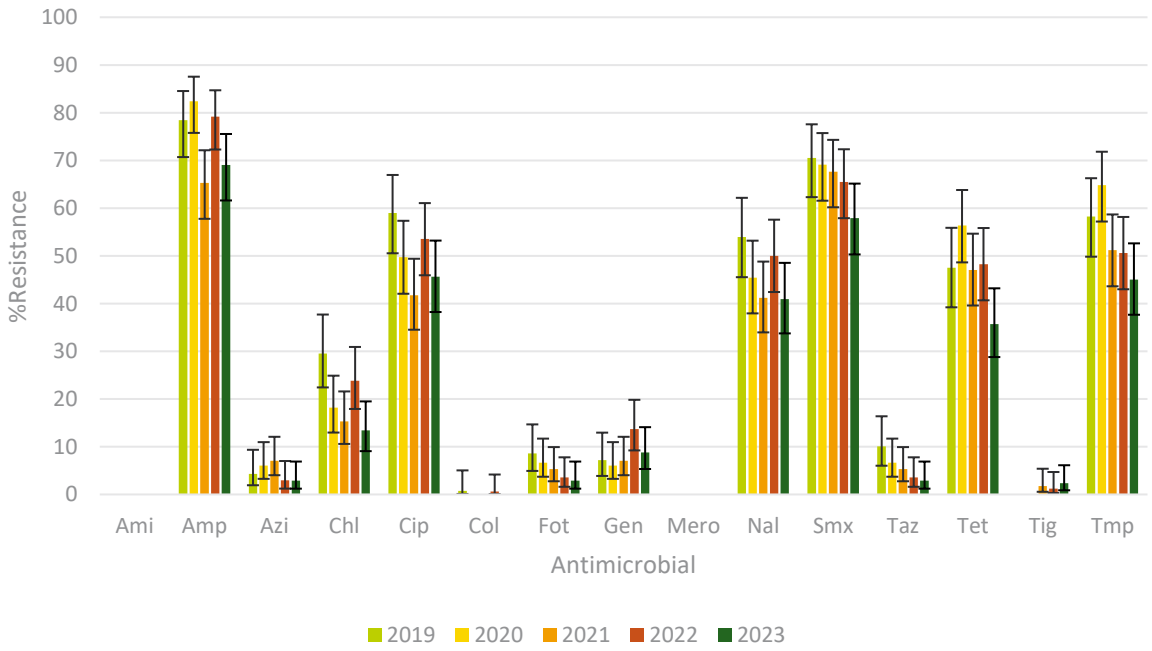

Figure 24. Trends in resistance rates to the first panel of antimicrobials in indicator *E. coli* isolated from broilers caeca (2019-2023).

In 2023, although Figure 24 shows a decrease in resistance to all antimicrobials apart from tigecycline, none of the results show a significant difference compared to previous years with the exception of resistance to chloramphenicol, which is significantly lower in 2023 compared to 2019.
Moreover, colistin resistance had been detected in 2022 and in 2019 but was not detected in 2023. We can also note the decreasing trend in resistance to 3rd generation cephalosporins (cefotaxime and ceftazidime) since 2019.
52.6% of the isolates were multi-drug resistant and 12.9% were susceptible to all antimicrobials tested. 5 isolates (2.9%) showed resistance to 3rd generation cephalosporins and were therefore tested with the second panel of antimicrobials (EUVSEC2). The results confirmed the ESBL phenotype according to EFSA classification.

3.2.7.2. Monitoring of commensal indicator *E. coli* in fattening pigs caecal content

180 samples of caecal content from fattening pigs were collected at the slaughterhouse for the detection of *E. coli* in 2023 and all were positive. 176 of those *E.coli* isolates were tested for antimicrobial susceptibility and the results are shown in Figure 25.

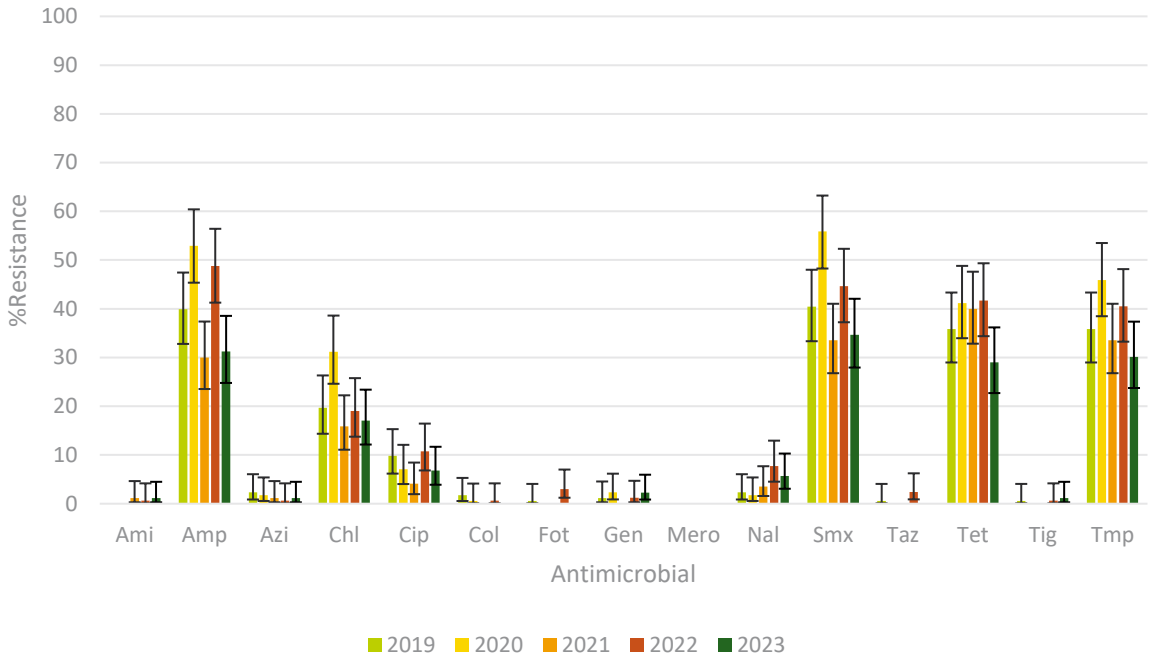

Figure 25. Trends in resistance rates to the first panel of antimicrobials in indicator *E. coli* isolated from fattening pigs caeca (2019-2023).

In comparison with 2022, Figure 25 shows a significant decrease in resistance to ampicillin. It is also important to note that in 2023, no resistance to 3rd generation cephalosporins was detected in commensal *E. coli* isolated from pig caecal content whereas this resistance was detected in 2022 and 2019. Resistance to colistin which was detected in 2019, 2020 and 2022 was also not detected in 2023. 2 isolates were resistant to amikacin in 2023. This resistance was detected in *E. coli* isolated from pig caecal content since the addition of this antimicrobial to the test panel in 2021.
Although not significant, a decreased resistance to chloramphenicol, ciprofloxacin, sulfamethoxazole, tetracycline and trimethoprim was detected in 2023 in comparison to 2022. However, resistances to azithromycin, gentamicin, and tigecycline slightly increased since 2022 but these changes were not significant either.

3.2.7.3. Monitoring of commensal indicator *E. coli* in bovine animals caecal content (slaughterhouse)

180 samples of caeca from bovines were taken at the slaughterhouse in 2022 for the detection of *E. coli* and were all positive. Of those, 17 were analysed by antimicrobial susceptibility testing. The results are shown in Figure 26.

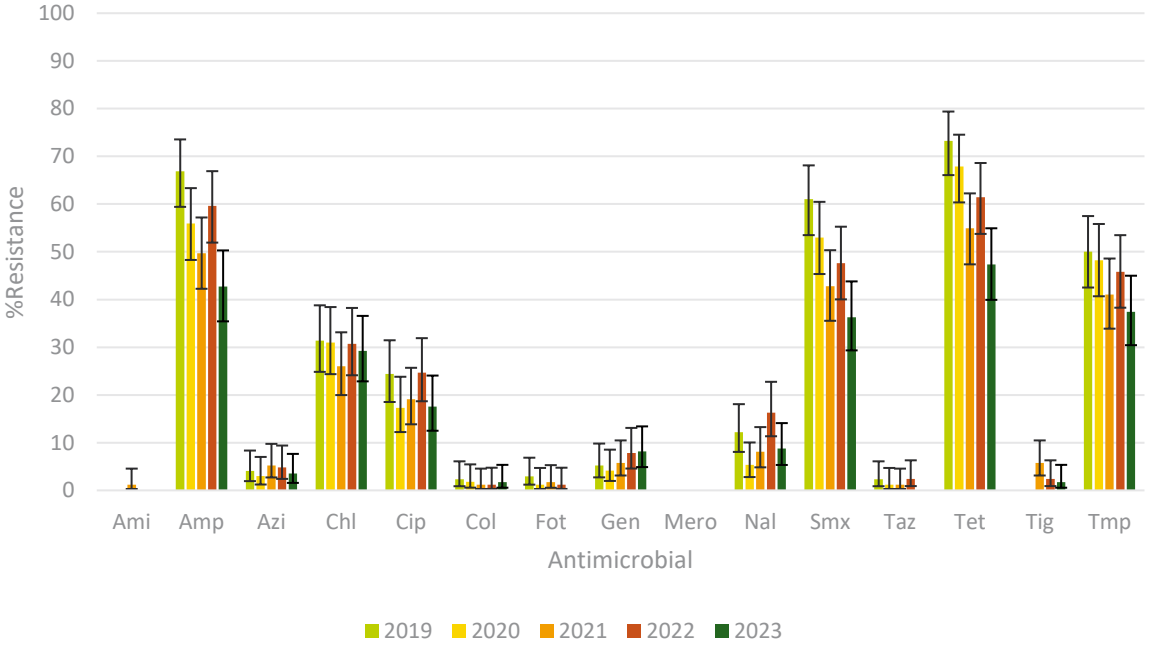

Figure 26. Trends in resistance rates to the first panel of antimicrobials in indicator *E. coli* isolated from bovines caeca (2019-2023).

In 2023, the only significant difference in the antimicrobial resistance of *E. coli* isolated from bovine caeca compared to 2022, similar to *E. coli* isolated from pig caeca, was the decreasing resistance to ampicillin.
There were small increases in resistance to gentamicin and colistin in 2023, but these remained at low levels. Otherwise, resistance to all antibiotics decreased in 2023 compared to 2022.
In addition, for the first time in all previous years, no resistance to 3rd generation cephalosporins was detected in 2023. No resistance to meropenem or amikacin was detected either.
41.5% of the isolates were multi-drug resistant and 44.4% were susceptible to all antimicrobials tested.

3.2.7.4. Monitoring of commensal indicator *E. coli* in faeces of bovine animals less than seven months old (farm)

In 2023, 177 samples of faeces from bovine animals less than seven months old were collected at farm level for the detection of indicator *E.coli* and all of them were positive. Of those, 171 samples were tested for antimicrobial susceptibility. The results are shown in Figure 27.

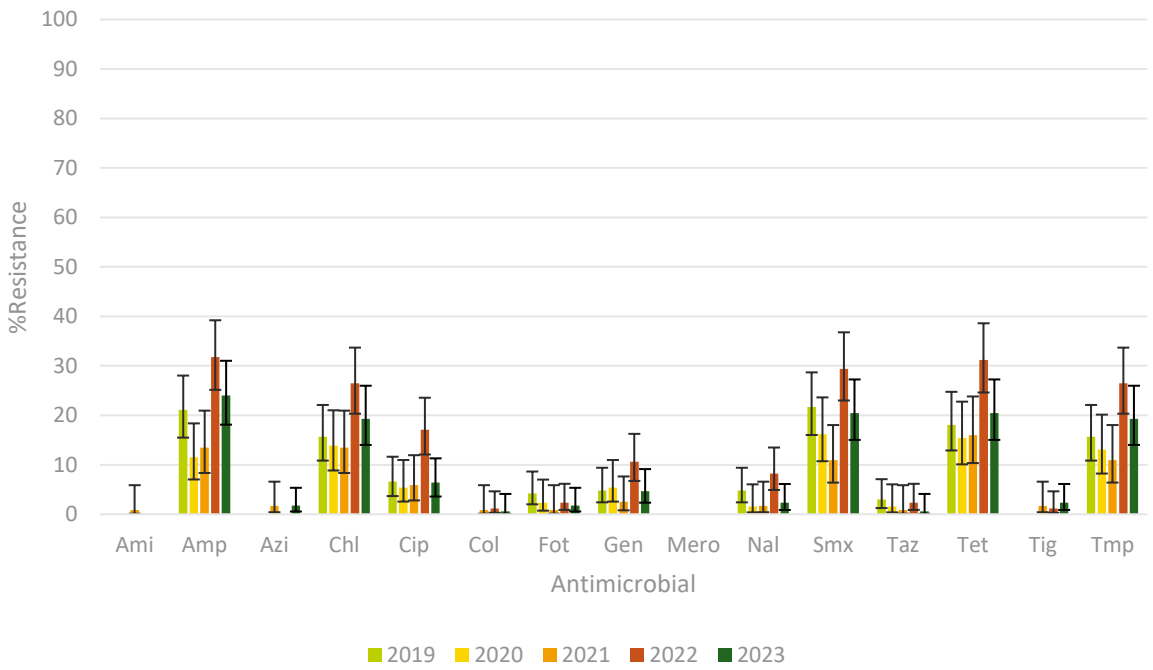

Figure 27. Trends in resistance rates to the first panel of antimicrobials in indicator *E. coli* isolated from bovines faeces (2019-2023).

After a significant increase in resistance to several antibiotics in 2022, most resistance levels decreased in 2023 although this decrease was only significant for ciprofloxacin (from 17.1% to 6%). However, resistance to tigecycline increased slightly in 2023 and resistance to azithromycin was detected again after not being detected in 2022.
Furthermore, 3 isolates (1.8%) showed resistance to 3rd generation cephalosporins. These 3 isolates were tested for the second panel of antimicrobials and the ESBL profile was confirmed with 2 of the 3 isolates showing an ESBL phenotype and the remaining one a combined ESBL + AmpC phenotype. No resistance to meropenem or amikacin was detected in 2023.
22.2% of the *E. coli* isolates were multi-drug resistant and 65.5% were susceptible to all antimicrobials tested.

3.2.7.5. Monitoring of commensal indicator *E. coli* in faeces from breeding and laying hens

In 2023, 169 fecal samples of breeding hens were collected at farm level for the detection of *E. coli*. 165 were positive and were analysed with antimicrobial susceptibility testing. The results are shown in Figure 28.

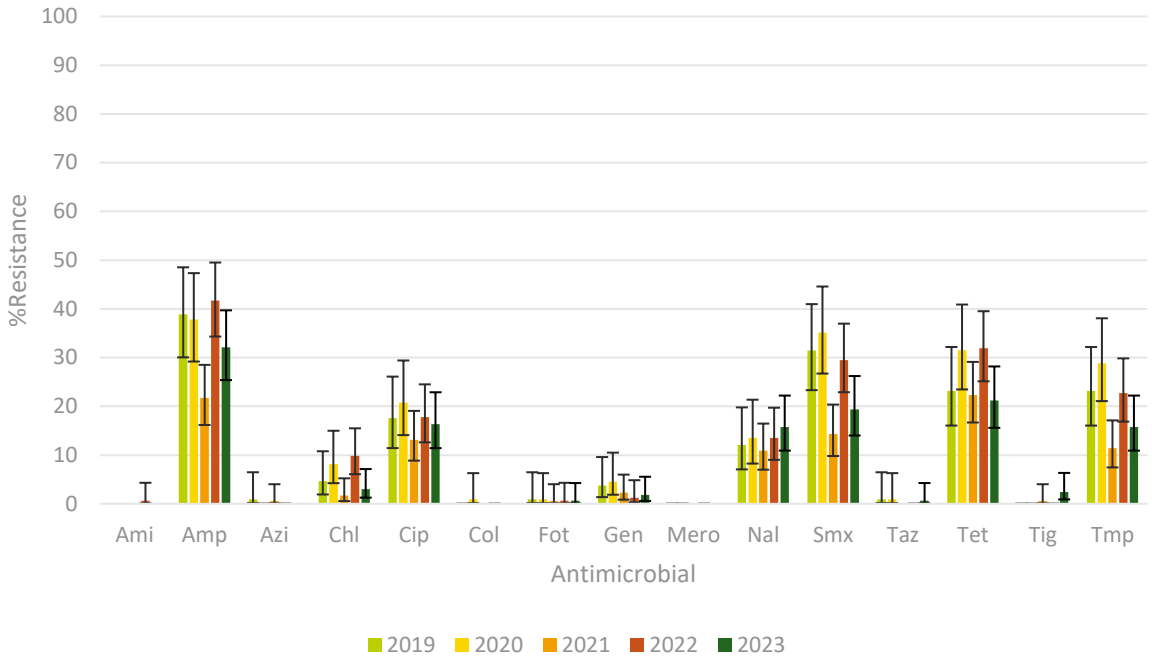

Figure 28. Trends in resistance rates to the first panel of antimicrobials in indicator *E. coli* isolated from faeces of breeding hens (2019-2023).

In 2023, no significant change in resistance to the different antimicrobials was detected. However, resistance to ceftazidime and to tigecycline were detected again after not being detected in 2022. We can also note that resistance to (fluoro)quinolones are on an increasing trend since 2020. Unlike in 2022, resistance to amikacin was not detected in 2023. Resistances to azithromycin, colistin or meropenem were not detected either.
One isolate (0.6%) was resistant to 3rd generation cephalosporins and was therefore tested with the second panel of antimicrobials which confirmed an ESBL phenotype.
29 isolates (17.6%) were multi-drug resistant and 81 (49.1%) were susceptible to all antimicrobials tested.

180 fecal samples of laying hens collected at farm level were also tested in 2023 for the detection of *E. coli.* 176 were positive and were tested for antimicrobial susceptibility. Results are shown in Figure 29.

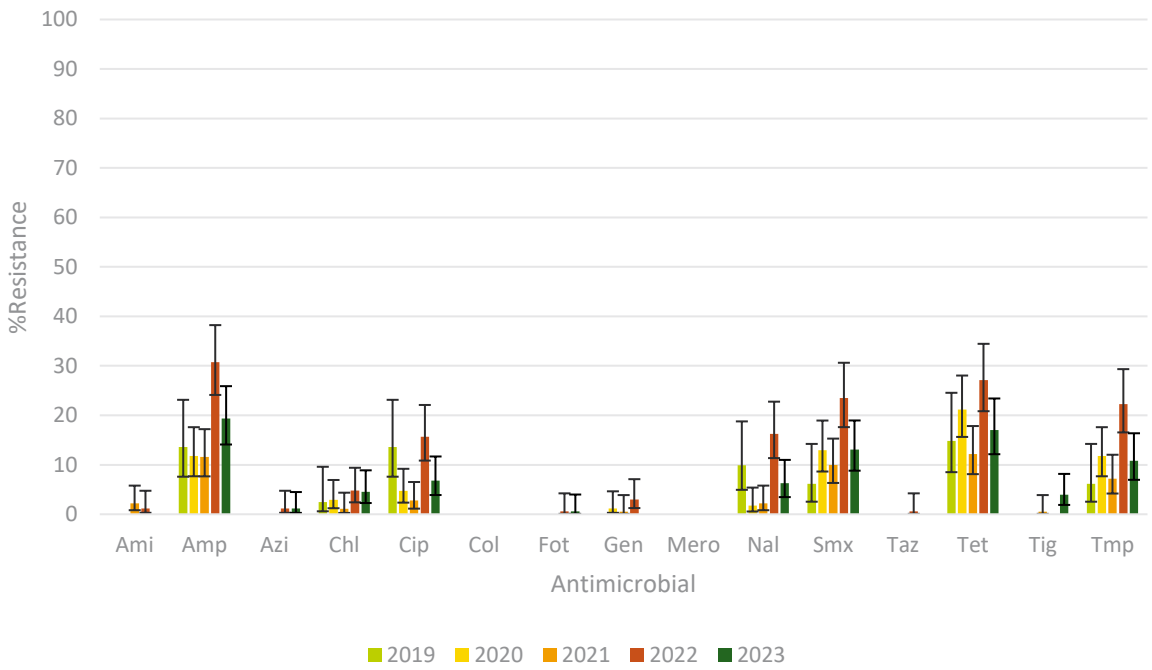

Figure 29. Trends in resistance rates to the first panel of antimicrobials in indicator *E. coli* isolated from faeces of laying hens (2019-2023).

After a significant increase in resistance to several antimicrobials was detected in 2022, they decreased in 2023 but remained higher than in 2021. Indeed, resistances to ampicillin, fluoroquinolones, tetracycline, sulfamethoxazole and trimethoprim decreased in 2023. 19.32% of the isolates were resistant to ampicillin, followed by tetracycline (17.1%), sulfamethoxazole (13.1%) and trimethoprim (10.8%). Seven isolates (4%) were resistant to tigecycline in 2023. Resistance to 3rd generation cephalosporins was also detected in one isolate in 2023. The results of the second panel of antimicrobials confirmed an ESBL phenotype. 2 isolates (1.1%) were also resistant to azithromycin in 2023. However, no resistance to meropenem or colistin was detected in 2023.

3.2.8. Specific monitoring of ESBL, AmpC or carbapenemases producing *E. coli*

3.2.8.1. Specific monitoring of ESBL, AmpC or carbapenemases producing *E. coli* in broilers caecal content

In 2023, as part of the specific search for *E. coli* bacteria producing ESBL, AmpC or carbapenemases present in the caecal contents of broilers, a qualitative method (detection/non-detection in 25g) was carried out. The medium used was MacConkey combined with ready-to-use cefotaxime (CTX, 1 mg/L) (Biorad) for the detection of ESBL and AmpC *E. coli* and CarbaSmart medium (BioMérieux) for the detection of carbapenemases.

290 samples of broilers caecal content were collected at the slaughterhouse and 179 were positive for ESBL/AmpC producing *E.* coli. There is therefore a small decrease in prevalence of ESBL producing *E. coli* from 78.54% in 2021, 75% in 2022 and 61.72% in 2023. No carbapenemases producing *E.coli* was detected. Whole genome sequencing was performed on 167 isolates.

3.2.8.1. Specific monitoring of ESBL, AmpC or carbapenemases producing *E. coli* in fattening pigs caecal content

In 2023, as part of the specific search for *E. coli* bacteria producing ESBL, AmpC or carbapenemases present in the caecal contents of fattening pigs, a qualitative method (detection/non-detection in 25g) was carried out. The medium used was MacConkey combined with ready-to-use cefotaxime (CTX, 1 mg/L) (Biorad) for the detection of ESBL and AmpC *E. coli* and CarbaSmart medium (BioMérieux) for the detection of carbapenemases.

311 samples of caecal content of fattening pigs were collected at the slaughterhouse for the detection of ESBL/AmpC producing *E.* coli. Of those, 91 were positive (29.26% compared to 34.94% in 2022) and 90 were tested for antimicrobial susceptibility. No carbapenemases producing *E.coli* was detected.

3.2.8.1. Specific monitoring of ESBL, AmpC or carbapenemases producing *E. coli* in bovine animals caecal content.

In 2023, as part of the specific search for *E. coli* bacteria producing ESBL, AmpC or carbapenemases present in the caecal contents of bovine animals, a qualitative method (detection/non-detection in 25g) was carried out. The medium used was MacConkey combined with ready-to-use cefotaxime (CTX, 1 mg/L) (Biorad) for the detection of ESBL and AmpC *E. coli* and CarbaSmart medium (BioMérieux) for the detection of carbapenemases.
312 samples of bovine animals caeca were collected at the slaughterhouse for the detection of ESBL/AmpC producing *E. coli*. 208 samples were positive (66.67% compared to 63.58% in 2022).

The Figure 30 illustrates the prevalence of ESBL *E. coli* during the period of time 2015-2023 for all three categories of food producing animals, broilers, pork and beef.

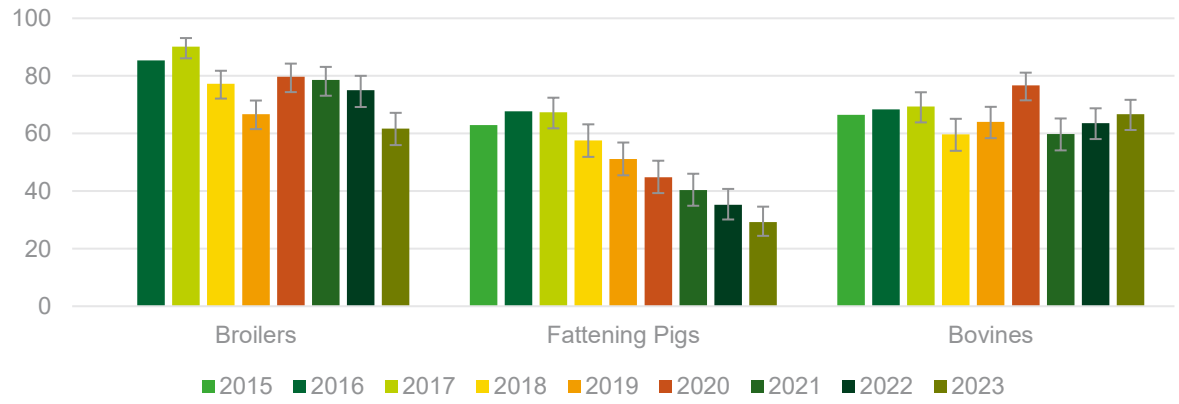

Figure 30. Prevalence of ESBL *E. coli* during 2015-2023 for all three categories of food producing animals, broilers, pork and beef.

Data analysis:
The antimicrobial prediction profile of ESBL *E. coli* isolates based on resistance genes detected by WGS, is illustrated in the Figure 31. It has been accepted that accurate prediction of AMR phenotypes according to ResFinder application v. 4.4.2; ResFinder Database v. 2.2.1; PointFinder Database v. 4.0.1 can replace the need to do antimicrobial susceptibility testing.
Results illustrate the AMR profile across the different sectors: broilers (PRI019), fattening pigs (PRI035) and bovines under 7 months (PRI036).
The predicted prevalence of *E. coli* producing extended spectrum β-lactamases based on the presence of at least one gene or chromosomal point mutation conferring resistance to cefotaxime and/or

ceftazidime was 97%, 90% and 98% on isolates from poultry, fattening pigs and bovines, respectively. None of the isolates were predicted to have resistance to meropenem.

Predicted ciprofloxacin resistance varied among isolates from the different categories of food producing animals, being the highest detected in isolates retrieved from broilers (57.49%), followed by bovines (38.19%) and fattening pigs (22.2%). The predicted prevalence of resistance to this antimicrobial is in line with resistance reported in previous years for broilers. After an increase observed in 2022, values from 2023 based on predicted prevalence are similar to those obtained in 2021, 56,99%. The predicted ciprofloxacin resistance in ESBL *E. coli* isolates from fattening pigs (22.2%) is similar to that reported in 2022 (24.04%). Regarding isolates from bovines, predicted resistance accounted for up to 38% in 2023 compared to 45% in 2022, year in which an increased was observed and 30.86% in 2021. Resistance to colistin was rare, it was predicted for one isolate from fattening pigs and one isolate from bovines. Considering that in 2022 resistance rate to azithromycin increased in ESBL *E. coli* isolates from all categories of food producing animals in 2023, predicted resistance was similar to that obtained in 2021 for all categories of animals except bovines. WGS has predicted azithromycin resistance for 24% of isolates from bovines, followed by 10% from fattening pigs and 8% from broilers. Those values reflect a decrease in the prevalence rate on isolates from broilers (13.74% in 2022) and fattening pigs (17.3% in 2022) and an increase on isolates retrieved from bovines (13.89% in 2022).

The distribution of extended spectrum β-lactamase genes conferring resistance to 3rd generation cephalosporins, cefotaxime and/or ceftazidime and to cefoxitin are illustrated in the Figure 31 and Table 20.

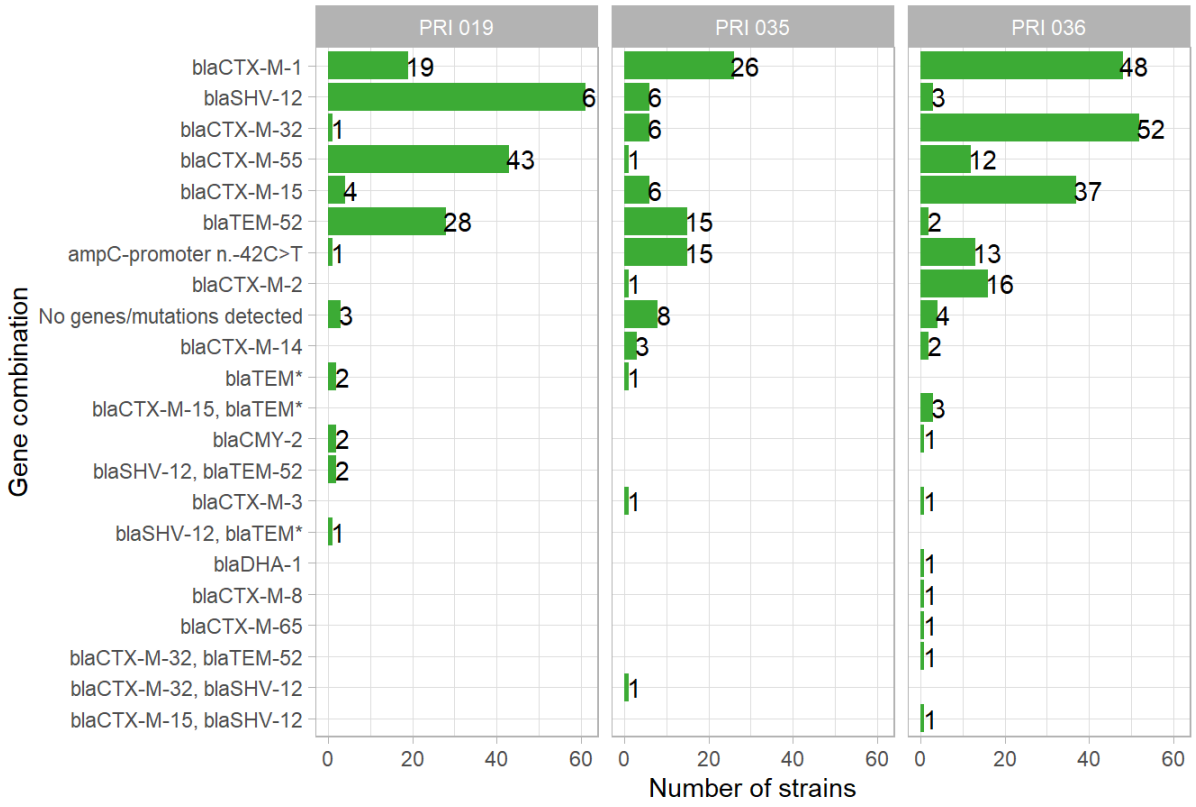

Figure 31. Distribution of resistant determinants inferring resistance to 3rd Generation cephalosporins.

Prevalence of ESBL genes differed among the different categories of animals. The predominant gene encoding for β-lactamases enzymes in isolates from broilers is *bla*$_{SHV-12}$ (36.53%, 61/167) followed by *bla*$_{CTX-M-55}$ (43/167, 25.75%) and other *bla*$_{CTX-M}$ genes encoding for CTX-M enzymes belonging to the group 1 (*bla*$_{CTX-M-1}$ and *bla*$_{CTX-M-15}$). In addition, the *bla*$_{TEM-52}$ was found in 61 isolates (16.77%). Two

isolates harbored the *bla*$_{CMY-2}$ and one isolate without any acquired resistance gene detected but with a point mutation in the ampC promoter (n.-42C>T). This mechanism of resistance is more frequently found in isolates from fattening pigs (15/90, 16.67%) and bovines (13/199, 6.53%). On those categories of food producing animals the predominant gene observed was *bla* $_{CTX-M-1}$ (26/90, 28.89%) in fattening pigs and *bla* $_{CTX-M-32}$ (52/199, 26.13%) in bovines, both belonging to the same CTX-M group 1. Other *bla*$_{CTX-M}$ variants have been observed as well, most of them belonging to the predominant CTX-M group 1, to note that the gene *bla*$_{CTX-M-2}$ encoding for enzyme production belonging to the group CTX-M 2, has been found in 16 isolates from bovines (8.04%). Results show that bovines could be a reservoir of CTX-M-2 producing *E.coli*. This gene has been found in isolates belonging to the same sequence type (ST488). For a limited number of isolates, no known gene/mutation associated to ESBL/AmpC phenotype could be predicted, in particular, 3 isolates from broilers, 8 from fattening pigs and 4 from bovines. Further research is ongoing.

Table 20. Distribution of resistant genes and mutations found in ESBL *E. coli* per category of food producing animals

gene	PRI 019 (n=167)	%	PRI 035 (n=90)	%	PRI 036 (n=199)	%	Total
bla$_{CTX-M-1}$	19	11,38	26	28,89	48	24,12	93
bla$_{SHV-12}$	61	36,53	6	6,67	3	1,51	70
bla$_{CTX-M-32}$	1	0,60	6	6,67	52	26,13	59
bla$_{CTX-M-55}$	43	25,75	1	1,11	12	6,03	56
bla$_{CTX-M-15}$	4	2,40	6	6,67	37	18,59	47
bla$_{TEM-52}$	28	16,77	15	16,67	2	1,01	45
ampC-promoter n.-42C>T	1	0,60	15	16,67	13	6,53	29
bla$_{CTX-M-2}$	0	0,00	1	1,11	16	8,04	17
bla$_{CTX-M-14}$	0	0,00	3	3,33	2	1,01	5
bla$_{TEM*}$	2	1,20	1	1,11	0	0,00	3
bla$_{CMY-2}$	2	1,20	0	0,00	1	0,50	3
bla$_{CTX-M-15}$, *bla*$_{TEM*}$	0	0,00	0	0,00	3	1,51	3
bla$_{CTX-M-3}$	0	0,00	1	1,11	1	0,50	2
bla$_{SHV-12}$, *bla*$_{TEM-52}$	2	1,20	0	0,00	0	0,00	2
bla$_{DHA-1}$	0	0,00	0	0,00	1	0,50	1
bla$_{CTX-M-8}$	0	0,00	0	0,00	1	0,50	1
bla$_{CTX-M-65}$	0	0,00	0	0,00	1	0,50	1
bla$_{CTX-M-15}$, *bla*$_{SHV-12}$	0	0,00	0	0,00	1	0,50	1
bla$_{CTX-M-32}$, *bla*$_{SHV-12}$	0	0,00	1	1,11	0	0,00	1
bla$_{CTX-M-32}$, *bla*$_{TEM-52}$	0	0,00	0	0,00	1	0,50	1
bla$_{SHV-12}$, *bla*$_{TEM*}$	1	0,60	0	0,00	0	0,00	1

Table 21. Proportion of isolates presenting a predicted phenotype, type ESBL and type AmpC

Predicted phenotype	PRI 019 (n=167)	%	PRI 035 (n=90)	%	PRI 036 (n=199)	%
ESBL	161	96,41	67	74,44	180	90,45
AmpC	3	1,80	15	16,67	15	7,54

ESBL *E. coli* resistant to fluoroquinolones

The distribution of plasmid mediated quinolone resistance genes (PMQR) and mutations in the quinolone resistance determining regions (QRDRs) are represented in the Figure 31 per each category of food producing animals.

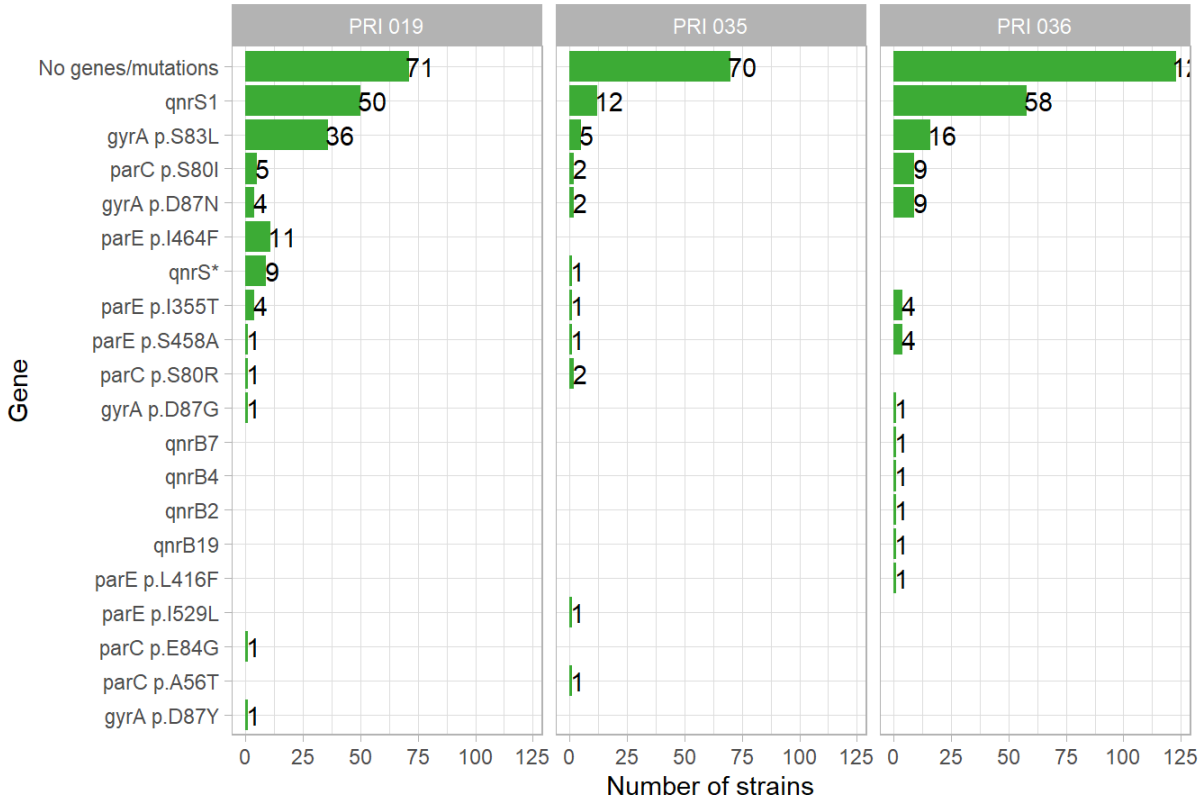

Figure 32. Distribution of resistant determinants inferring resistance to (fluoro)quinolones

Among the ESBL *E. coli*, predicted sensibility to ciprofloxacin was observed for 42.5% (71/167), 77.7% (70/90) and 61.81% (123/199) from poultry, fattening pigs and bovines respectively. Predicted resistance to (fluoro)quinolones considering all the resistance genes together with the chromosomal mutations was observed in 57.49%, 22.22%, and 38.12% in isolates from broilers, fattening pigs and bovines respectively.

The gene most frequently found was *qnrS* in broilers (29.94% 50/167), followed by bovines (29.15%, 58/167) and by fattening pigs (13.33%, 12/90). The second most prevalent mechanism of resistance found was mutations in the quinolone resistance determining regions (QRDRs) in chromosomal *gyrA* gene in 21.56%, 8.04% and 5.56% of isolates from broilers, bovines, and fattening pigs respectively.

The combination of genes and mutations predicting an ESBL/AmpC phenotype and those predicting resistance to fluoro(quinolones) are presented in the Table 22.

The gene *qnrS* is frequently associated to *bla*$_{\text{CTX-M}}$ group 1, the most prevalent association found in broilers was *bla*$_{\text{CTX-M-55}}$/*qnrS,* in fattening pigs *bla*$_{\text{CTX-M-1-15/}}$ *qnrS*, and in bovines *bla*$_{\text{CTX-M-15/}}$ *qnrS*.

The gene *qnrS* was present as well in isolates from broilers harboring *bla*$_{\text{SHV-2}}$.

Table 22. Combination of resistant determinants inferring resistance to (fluoro)quinolones in ESBL *E. coli*

	PRI 019	%	PRI 035	%	PRI 036	%
bla$_{\text{CTX-M-55}}$, *qnrS1*	43	44,79	0	0	1	1,27
bla$_{\text{CTX-M-15}}$, *qnrS1*	2	2,08	5	23,81	21	26,58
bla$_{\text{CTX-M-1}}$, *qnrS1*	0	0,00	5	23,81	11	13,92
bla$_{\text{CTX-M-32}}$, *qnrS1*	0	0,00	1	4,76	12	15,19
bla$_{\text{SHV-12}}$, *qnrS**	9	9,38	1	4,76	0	0,00

bla$_{TEM-52}$, *gyrA p.S83L, parE p.I464F*	10	10,42	0	0,00	0	0,00
bla$_{SHV-12}$, *gyrA p.S83L*	8	8,33	0	0,00	0	0,00
bla$_{CTX-M-15}$, *gyrA p.D87N, gyrA p.S83L, parC p.S80I, parE p.S458A*	1	1,04	1	4,76	2	2,53
bla$_{CTX-M-2}$, *qnrS1*	0	0,00	0	0,00	4	5,06
bla$_{CTX-M-55}$, *gyrA p.D87N, gyrA p.S83L, parC p.S80I, qnrS1*	0	0,00	0	0,00	4	5,06
bla$_{SHV-12}$, *qnrS1*	4	4,17	0	0,00	0	0,00
bla$_{CTX-M-1}$, *gyrA p.S83L*	3	3,13	0	0,00	0	0,00
bla$_{CTX-M-32}$, *gyrA p.S83L*	0	0,00	0	0,00	3	3,80
ampC-promoter n.-42C>T, gyrA p.D87N, gyrA p.S83L, parC p.S80I	1	1,04	1	4,76	0	0,00
bla$_{CTX-M-1}$, *gyrA p.D87N, gyrA p.S83L, parC p.S80I*	0	0,00	0	0,00	2	2,53
bla$_{CTX-M-1}$, *gyrA p.S83L, parE p.I355T*	2	2,08	0	0,00	0	0,00
bla$_{CTX-M-15}$, *blaTEM**	0	0,00	0	0,00	2	2,53
bla$_{CTX-M-32}$, *parE p.I355T*	0	0,00	0	0,00	2	2,53
bla$_{SHV-12}$, *blaTEM-52, gyrA p.S83L*	2	2,08	0	0,00	0	0,00
bla$_{SHV-12}$, *gyrA p.S83L, parC p.S80R*	1	1,04	1	4,76	0	0,00
bla$_{TEM-52}$, *gyrA p.S83L*	2	2,08	0	0,00	0	0,00
ampC-promoter n.-42C>T, gyrA p.S83L	0	0,00	0	0,00	1	1,27
ampC-promoter n.-42C>T, parE p.I355T	0	0,00	1	4,76	0	0,00
bla$_{CTX-M-1}$, *gyrA p.D87G, gyrA p.S83L, parC p.S80I, qnrS1*	1	1,04	0	0,00	0	0,00
bla$_{CTX-M-1}$, *gyrA p.D87N, gyrA p.S83L, parC p.E84G, parC p.S80I, parE p.I355T*	1	1,04	0	0,00	0	0,00
bla$_{CTX-M-1}$, *qnrB7*	0	0,00	0	0,00	1	1,27
bla$_{CTX-M-14}$, *gyrA p.S83L, parC p.S80R*	0	0,00	1	4,76	0	0,00
bla$_{CTX-M-15}$, *blaSHV-12, qnrS1*	0	0,00	0	0,00	1	1,27
bla$_{CTX-M-15}$, *blaTEM*, qnrS1*	0	0,00	0	0,00	1	1,27
bla$_{CTX-M-15}$, *gyrA p.D87N, gyrA p.S83L, parC p.S80I, parE p.L416F, qnrS1*	0	0,00	0	0,00	1	1,27
bla$_{CTX-M-15}$, *parE p.I355T*	0	0,00	0	0,00	1	1,27
bla$_{CTX-M-32}$, *blaSHV-12*	0	0,00	1	4,76	0	0,00
bla$_{CTX-M-32}$, *blaTEM-52*	0	0,00	0	0,00	1	1,27
bla$_{CTX-M-32}$, *qnrB19*	0	0,00	0	0,00	1	1,27
bla$_{CTX-M-55}$, *gyrA p.D87G*	0	0,00	0	0,00	1	1,27
bla$_{CTX-M-55}$, *gyrA p.S83L*	0	0,00	0	0,00	1	1,27
bla$_{CTX-M-55}$, *gyrA p.S83L, parE p.S458A*	0	0,00	0	0,00	1	1,27
bla$_{CTX-M-55}$, *gyrA p.S83L, parE p.S458A, qnrS1*	0	0,00	0	0,00	1	1,27
bla$_{CTX-M-65}$, *qnrS1*	0	0,00	0	0,00	1	1,27
bla$_{DHA-1}$, *parE p.I355T, qnrB4*	0	0,00	0	0,00	1	1,27
bla$_{SHV-12}$, *blaTEM*, gyrA p.S83L*	1	1,04	0	0,00	0	0,00
bla$_{SHV-12}$, *gyrA p.D87N, gyrA p.S83L, parC p.S80I*	1	1,04	0	0,00	0	0,00
bla$_{SHV-12}$, *gyrA p.D87Y*	1	1,04	0	0,00	0	0,00
bla$_{SHV-12}$, *parC p.A56T*	0	0,00	1	4,76	0	0,00
bla$_{SHV-12}$, *parE p.I355T*	1	1,04	0	0,00	0	0,00
bla$_{SHV-12}$, *qnrB2*	0	0,00	0	0,00	1	1,27
bla$_{TEM*}$, *qnrS1*	0	0,00	1	4,76	0	0,00

bla$_{TEM-52}$, *gyrA p.S83L, parE p.I529L*	0	0,00	1	4,76	0	0,00
gyrA p.S83L	1	1,04	0	0,00	0	0,00
gyrA p.S83L, parE p.I464F	1	1,04	0	0,00	0	0,00

ESBL *E. coli* resistant to azithromycin

Predicted resistance to azithromycin is rare. No resistance genes have been found in 91.62 % of the isolates from broilers, followed by 90% on fattening pigs and 78.38% in bovines.
The most frequently found gene was *mph(A)* in 8.3%, 10% and 18.59% of isolates from broilers, pigs and bovines respectively.

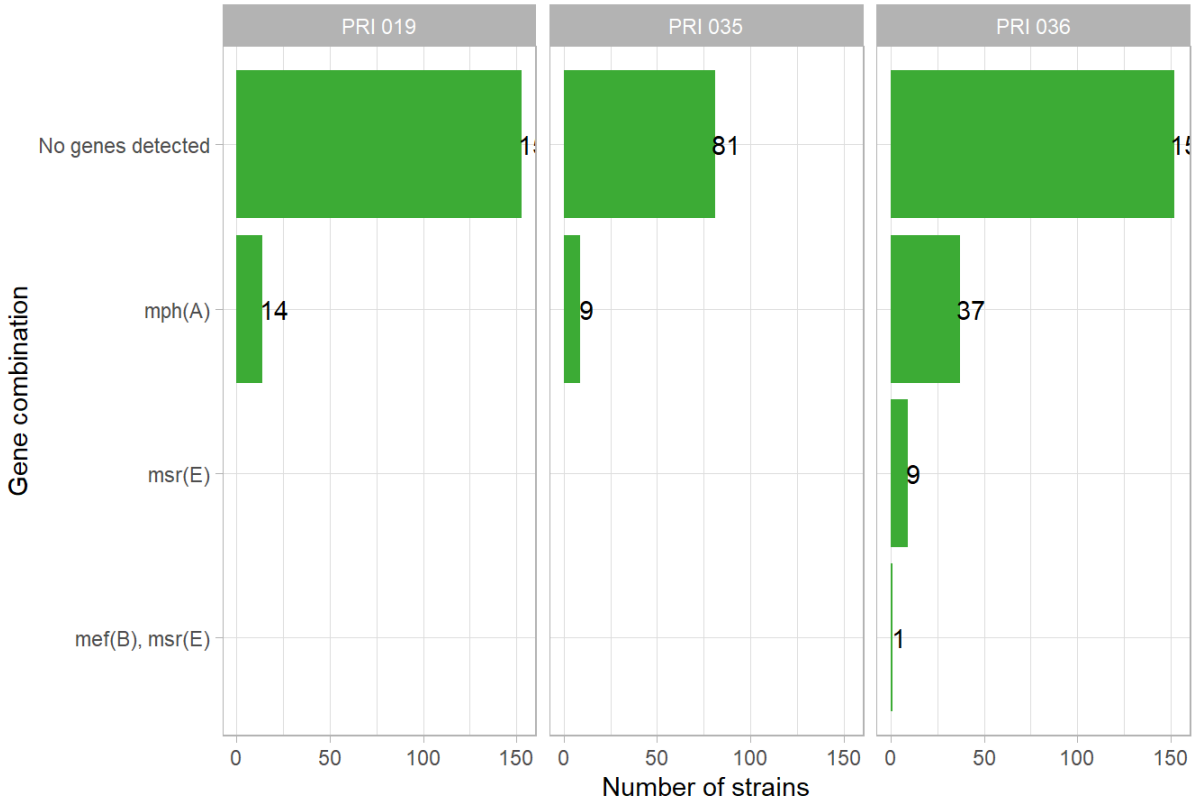

Figure 33. Distribution of determinants inferring resistance to azithromycin

ESBL *E. coli* resistant to colistin

Resistance to colistin is rare. Predicted sensitivity to this antimicrobial was detected in 100%, 98,88% and in 98,99% of isolates from broilers, fattening pigs and bovines. Three acquired resistance genes associated to phenotypic resistance to colistin were found. The *mcr 2.1* was found in one isolate from fattening pigs and the other isolates harboring a *mcr 1.1* gene were recovered from bovines.
The isolate from fattening pigs harbored in addition to the *mcr 2.1* gene, the following genes as well, *bla*$_{CTX-M-1}$, *bla*$_{TEM-1}$, *aph(3'')-Ib*, *aadA5, sul2, tet(M)* confering resistance to extended spectrum β-lactams, and *the* aminoglycosides, spectinomycin and streptomycin, sulfamethoxazole and tetracycline.
The two isolates from bovines belonging to the same ST540 carried in addition to the *mcr 1.1*, the following genes as well, *bla*$_{TEM-135}$, *cmlA1, sul3, tet(A), tet(M), dfrA8* confering a multidrug resistance profile comprising, β-lactams, chloramphenicol, sulfamethoxazole, tetracycline and trimethoprim.

ESBL *E. coli* resistant to gentamicin

Predicted sensitivity for gentamicin has been detected in 76%, 95% and 77.39% of isolates from broilers, fattening pigs and bovines. The most frequent gene found in ESBL *E. coli* isolates was *aac(3)-IId* in

22.15% and 14.57% from broilers and bovines, respectively. All the genes found associated with gentamicin resistance are illustrated in the Figure 34.

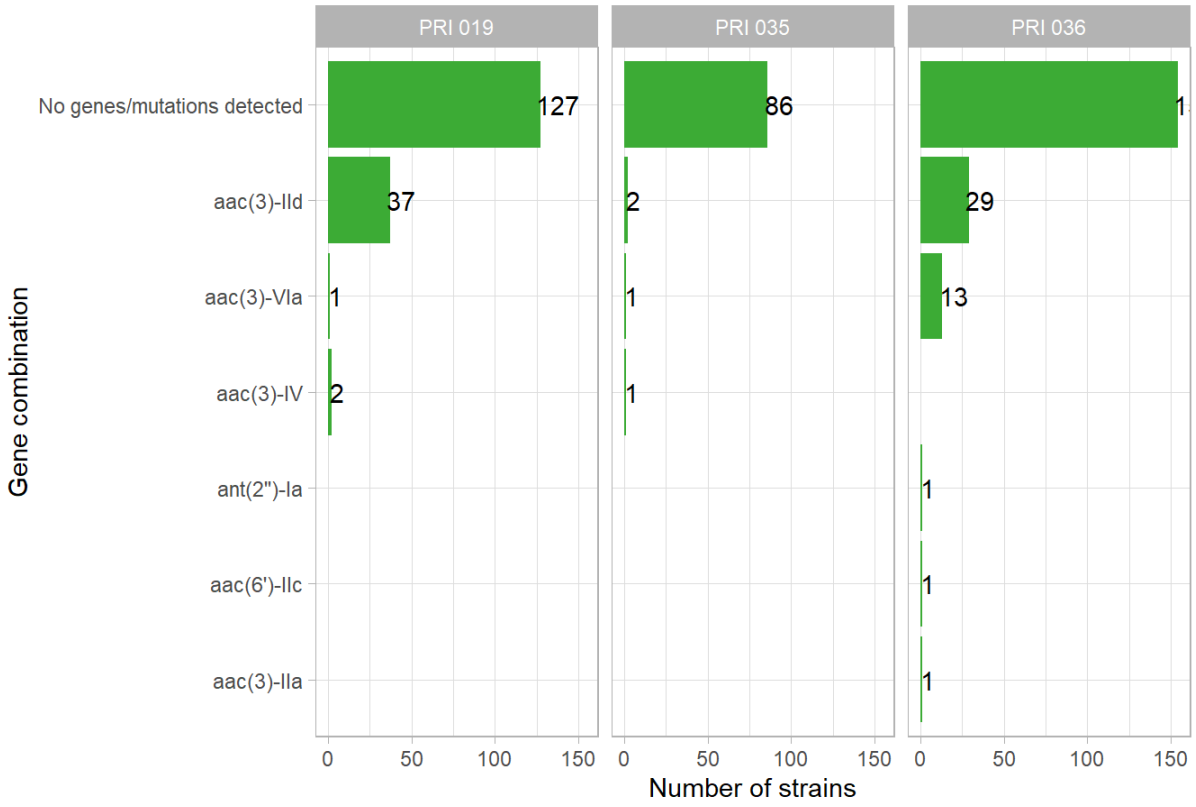

Figure 34. Distribution of determinants inferring resistance to gentamicin

3.2.9. Antimicrobial resistance monitoring of methicillin-resistant *Staphylococcus aureus* isolated from broilers, laying hens and fattening turkeys

The monitoring of MRSA in bovines, pigs and poultry has been repeated every three years starting in 2011 with poultry, in 2012 with bovines and in 2013 with pigs. The distinction between fattening pigs and sows was reported since 2016. In 2023, samples were taken of broilers, laying hens and fattening turkeys in order to assess the MRSA prevalence in these animal categories and determine the genotypes (STs and *spa*-types) of 6 of collected MRSA isolates together with their AMR and virulence genes. The antimicrobial resistance of MRSA was only studied genetically and no more phenotypically (no susceptibility testing) in 2023.

3.2.9.1. Prevalence of methicillin-resistant *Staphylococcus aureus* in broilers, laying hens and fattening turkeys

In 2023, an MRSA prevalence of 0.0% (n=0/33, Ci95% [0.0-10.4%]) in laying hens, 1.0% (n=1/96, Ci95% [0.2-5.7%] in broilers and 18.5% (n=5/27, Ci95% [8.2-36.7%]) in fattening turkeys was observed in Belgium with the 1-S isolation method (see Table 23). The MRSA prevalence remains stable since 2011 in broiler and laying hens and compared to 2020 (first year of turkey monitoring) in fattening turkeys. All the 6 isolates collected in 2023 were confirmed to belong to the *S. aureus* species and carried the *mecA* gene.

Table 23. Prevalence of MRSA in broilers, laying hens and fattening turkeys according to the year and the isolation method.

Animal category	Year	Isolation method	N samples	N positive	% positive	CI 95%
Broilers	2023	1-S	96	1	**1.0%**	0.2-5.7%
	2020	2-S	60	2	**3.3%**	0.9-11.4%
	2017	2-S	80	2	**2.5%**	0.7-8.7%
	2014	2-S	79	2	**2.5%**	0.7-8.8%
	2011	2-S	92	3	**3.3%**	1.1-9.1%
Laying hens	2023	1-S	33	0	**0.0%**	0.0-10.4%
	2020	2-S	28	0	**0.0%**	0.0-12.1%
	2017	2-S	236	3	**1.3%**	0.4-3.7%
	2014	2-S	246	6	**2.4%**	1.1-5.2%
	2011	2-S	280	0	**0.0%**	0.0-1.3%
Fattening turkeys	2023	1-S	27	5	**18.5%**	8.2-36.7%
	2020	2-S	18	2	**11.1%**	3.1-32.8%

3.2.9.2. WGS investigation of methicillin-resistant *Staphylococcus aureus*

Among the 156 samples collected in 2023 in Belgium, 6 MRSA (n= 5 from fattening turkeys and n=1 from broilers) were isolated and analysed by whole genome sequencing.

- **Genotyping : STs and *spa*-types**

The single MRSA isolate detected in broilers belonged to ST398 and t011 *spa*-type, typical of Livestock associated MRSA (LA-MRSA). Among the 5 MRSA isolated from fattening turkeys, 3 belonged also to the ST398-t011 lineage. The 2 remaining turkey isolates belonged to the spa-type t011 and to the new sequence type ST8731. This ST8731 is a single locus variant of ST398 and was thus considered to belong to CC398 LA-MRSA clonal complex, according to the rules described in Feil *et al.* (2004).

- **Antimicrobial resistance genes**

All the 6 MRSA isolates collected in 2023 belonged to CC398 and were harboring the *mecA* gene mediating methicillin resistance and the *tet(M)* tetracycline resistance gene, which is typical of LA-MRSA. Besides *tet(M)*, *tet(38)* was also observed in all the isolates, *tet(K)* in 2 isolates from turkeys and

tet(L) in 2 other isolates from turkeys. In addition, with the exception of one turkey isolate, all were characterized by the presence of *blaZ* conferring resistance to penicillin.
A diaminopyrimidines resistance gene was observed in all the isolates (either *dfrK* in 4 isolates from turkeys and 1 from broilers or *dfrG* in one isolate from turkeys).
The *erm(C)* gene encoding macrolides-lincosamides-streptogramins resistance was observed alone (n=2 fattening turkeys, n=1 broilers) or in combination with *erm(T)* (n=2 fattening turkeys). The last isolate from turkeys carried the *lnu(B)* and *lsa(E)* genes, encoding resistance to lincosamides alone, or lincosamides, pleuromutilins and streptogramins, respectively.
Aminoglycosides resistance genes were observed in 4 out of the 5 turkeys isolates (*aac(6')-aph(2")* (n=3, one of which is truncated and thus potentially not functional), *aadD* (n=2), *spd* (n=2) or *ant(9)-Ia* (n=1, truncated)). One of these turkey isolates carried only a truncated aminoglycoside resistance gene (*ant(9)-Ia*), phenotypic resistance to aminoglycosides would therefore be interesting to assess for this isolate specifically. The broiler isolate carried the *spd* gene.
No gene encoding oxazolidinone resistance neither vancomycin resistance was detected in the MRSA strains isolated in 2023.
All the 6 MRSA isolates were genetically multi-drug resistant (i.e., carrying genes conferring resistance to at least 3 different antibiotic classes), (see Annex Ia).

- **Biocide resistance genes**

In addition to the detection of antimicrobial resistance genes, the ResFinder tool used allow also the detection of some biocide resistance genes. None of them were observed in MRSA in 2023 (see Annex Ib).

- **Virulence genes**

In 2023, several virulence genes were observed among the collection of MRSA isolates from broilers and fattening turkeys: genes associated with toxins (*selw, hlgA, hlgB* and *hlgC*), genes associated with the human immune evasion cluster (*sak, scn*) and exoenzyme (*aur*) (see Annex Ic). At least four virulence genes were detected in all the 6 isolates: *aur, selw, hlgB* and *hlgC*. The toxins associated gene *hlgA* was detected in all but one isolates (absent in one ST398/t011 turkey isolate). The toxins associated *seb* gene was not detected. In addition, one fattening turkey isolate belonging to ST398/t011 genotype harbored the two *sak* and *scn* genes associated with the immune evasion cluster. The genes associated with the immune evasion cluster were not observed in the other isolates.
The *aur* gene encodes the aureolysin, a typical exoenzyme from *S. aureus* (Dubin, 2022); the *selw* gene was already reported to be commonly found in CC398 MRSA (Vrieling et al, 2020); the *hlgA*, *hlgB* and *hlgC* are common in Belgian LA-MRSA. (see Annex Ic)

3.2.9.3. Discussion

The presence of MRSA in food-producing animals and their carriage of several AMR and virulence genes represents a public health risk. In 2023, the MRSA prevalence was very low in broilers and moderate in fattening turkeys while null in laying hens (stable trends). All the 6 MRSA isolates collected (5 from fattening turkeys and one from broilers) and analysed were genetically multi-drug resistant (i.e., carrying genes conferring resistance to at least 3 different antibiotic classes). However, in 2023, no gene encoding resistance to the critically important antibiotics (linezolid and vancomycin) was detected. All the 6 MRSA isolates collected in 2023 belonged to CC398 and were harboring the *mecA* gene mediating methicillin resistance and the *tet(M)* tetracycline resistance gene, which is typical of LA-MRSA. One LA-MRSA isolate from fattening turkeys carried the *sak* and *scn* genes associated with the human immune evasion cluster and several genes associated with toxins (*hlgA*, *hlgB*, *hlgC*, and *selw*) and exoenzyme (*aur*). Cet isolat n'a probablement pas été récemment transmis par l'homme à la dinde, étant donné le contexte génétique associé au bétail et le portage du gène *tet(M)*. The genes associated with the immune evasion cluster were not observed in the other isolates.

Several changes in the methodology used for the monitoring of MRSA have been made in 2022 and still applied in 2023, including a new isolation method and the study of AMR through WGS rather than

phenotypic susceptibility testing. The previous and current isolation method were compared in the past in Belgium for poultry (layers and broilers) and cattle and this comparison did not detect significant differences in the performance of the two methods for these animal categories in Belgium (Nemeghaire et al., 2013 and 2014). Therefore, we can assume that the trends can be assessed from the entire period (whatever the isolation method used) for poultry and cattle. A point to bear in mind when analyzing AMR through the detection of genes rather than through phenotypic testing is that these genes could not be expressed. Thus, the detection of a gene does not imply that a phenotypic resistance would be observed for the corresponding antibiotic. On the other hand, genetic data provides key information on the way an AMR trait could be (or not) transmitted and to follow in the future monitoring the main genetic drivers of AMR.

3.2.10. Antimicrobial resistance monitoring of *Enterococcus faecium* and *Enterococcus faecalis* isolated from broilers, breeders, layers, pigs and veal calves faeces

3.2.10.1. Prevalence of *Enterococcus faecalis* and *Enterococcus faecium* isolated from broiler, breeder, layer, pig and veal calve faecal samples

From 266 broiler samples, *E. faecalis* was isolated in 183 samples (68.8%) and *E. faecium* in 138 samples (51.9%). Among 181 breeder samples and 218 layer samples, *E. faecium* was more often isolated than *E. faecalis*, with 160 (88.4%) and 157 (72.0%) samples positive for *E. faecium* against 53 (29.3%) and 87 (39.9%) positive for *E. faecalis*, respectively. Among 252 veal calve samples, 138 (54.8%) *E. faecium* and 90 *E. faecalis* (35.7%) were isolated. From 268 pig samples, *E. faecium* was isolated up to 3 times more often than *E. faecalis* with n=167 (62.3%) and n=56 (20.9%) respectively (see Figure 35).

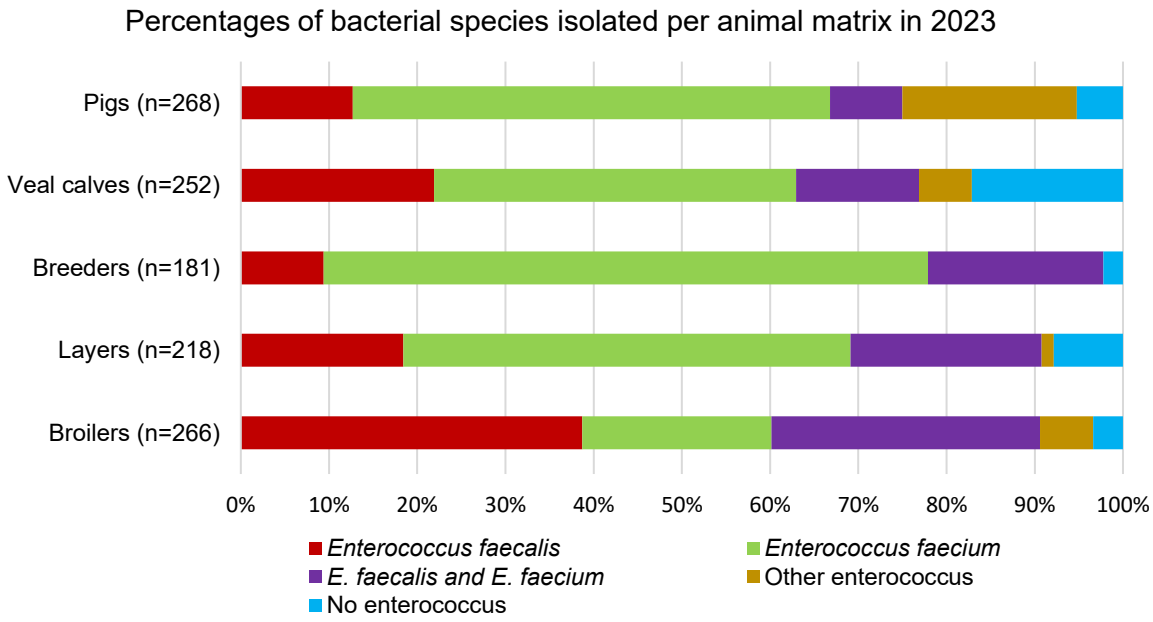

Figure 35. Prevalence of *Enterococcus faecalis* and *Enterococcus faecium* isolation per animal matrix in 2023.

3.2.10.2. Antimicrobial resistance in *Enterococcus faecium* and *Enterococcus faecalis* isolated from broiler, breeder and layer faecal samples

- Broiler samples collected at the slaughterhouse

A total of 320 antimicrobial susceptibility tests were performed after isolation of *Enterococcus faecalis* (n=182) and *Enterococcus faecium* (n=138) from broiler samples.
Within these samples, very high to extremely high levels of resistance were observed for erythromycin, quinupristin/dalfopristin and tetracycline. Indeed, 81.9% of *E. faecalis* and 69.6% of *E. faecium* showed resistance to tetracycline; as well as 78.6% of *E. faecalis* and 69.6% of *E. faecium* with erythromycin resistance; and 77.5% of *E. faecium* isolates resistant to quinupristin/dalfopristin. Moreover, the occurrence of ampicillin resistance was moderate, observed in 17.4% of *E. faecium*, only. The level of resistance observed to ciprofloxacin (5.8%) was low in *E. faecium*. Resistance to gentamicin was very low (0.7%) in *E. faecium* and low (1.6%) in *E. faecalis*. In addition, chloramphenicol resistance was observed in a very low level in both *E. faecalis* and *E. faecium*.
No resistance to daptomycin, linezolid, teicoplanin, tigecycline or vancomycin was observed in broilers in 2023 (see Figure 36).

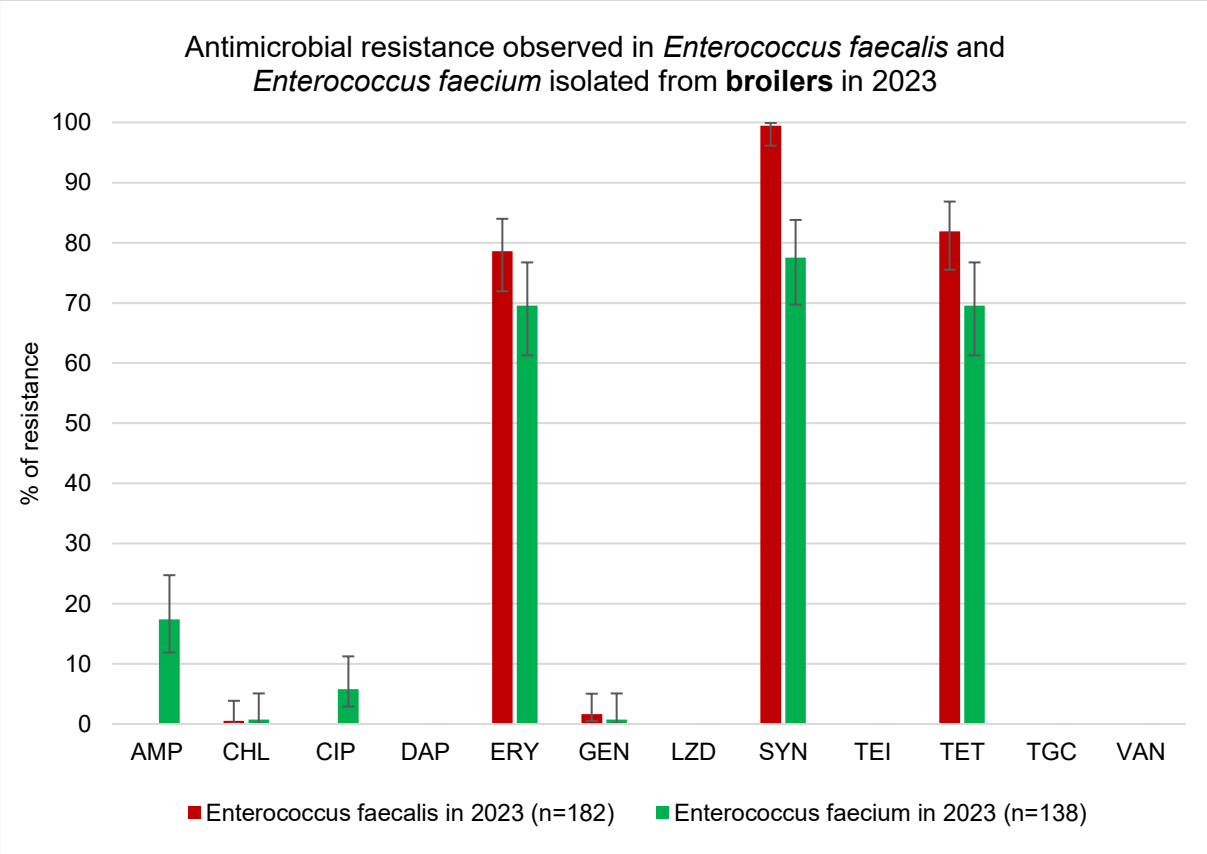

Figure 36. Percentages of antimicrobial resistance in *Enterococcus faecalis* (n=182) and *Enterococcus faecium* (n=138) isolated from broilers in 2023.
Based on epidemiological cut-offs according to the European Committee on Antimicrobial Susceptibility (EUCAST) for the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC) and vancomycin (VAN).

- Breeder samples collected at farm

A total of 213 antimicrobial susceptibility tests were performed after isolation of *Enterococcus faecalis* (n=53) and *Enterococcus faecium* (n=160) from breeder samples.

In breeding hens samples, very high resistance to tetracycline (52.8%) and low resistance to erythromycin (9.4%) were observed in *E. faecalis*. In *E. faecium*, an extremely high rate of resistance to quinupristin/dalfopristin (80.0%) was observed as well as a high rate to tetracycline (44.4%) and a moderate rate to erythromycin (15.0%). A moderate level of resistance to ampicillin (11.9%) was also observed in *E. faecium* only, this resistance being absent in *E. faecalis*. In addition, a low resistance rate to ciprofloxacin (5.6%) and a very low resistance rate to chloramphenicol (0.6%) were observed in *E. faecium*.
A low rate of resistance to daptomycin was observed in *E. faecalis* (1.9%), this resistance was absent in *E. faecium*. No resistance to gentamicin, linezolid, teicoplanin, tigecycline or vancomycin was observed in breeders in 2023 (see Figure 37).

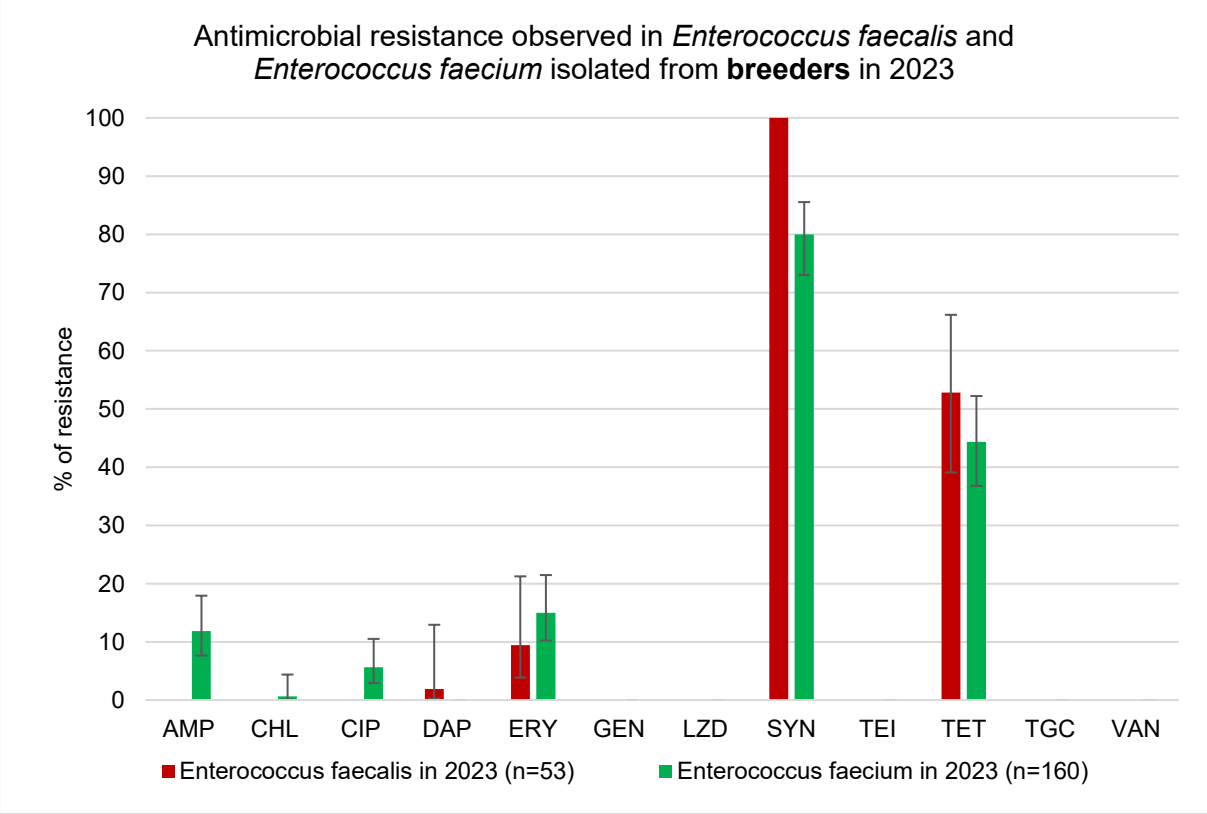

Figure 37. Percentages of antimicrobial resistance in *Enterococcus faecalis* (n=53) and *Enterococcus faecium* (n=160) isolated from breeders in 2023.
Based on epidemiological cut-offs according to the European Committee on Antimicrobial Susceptibility (EUCAST) for the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC) and vancomycin (VAN).

- Layer samples collected at farm

A total of 243 antimicrobial susceptibility tests were performed after isolation of *Enterococcus faecalis* (N=87) and *Enterococcus faecium* (N=156) from laying hens samples.
A very high rate of resistance to tetracycline (51.7%) and high to erythromycin (25.3%) was observed in *E. faecalis*. Within *E. faecium* isolates, a high rate of resistance to quinupristin/dalfopristin (48.7%), as well as a moderate rate to erythromycin (16.0%) and a low rate to tetracycline (9.0%) were observed. Moreover, a low rate of resistance to chloramphenicol was also found in *E. faecalis* only (1.1%). On the contrary, low levels of resistance to ampicillin (2.6%) and to ciprofloxacin (1.9%) were observed in *E. faecium* only.
A low rate of resistance to daptomycin was observed in *E. faecalis* (1.1%), this resistance was absent in *E. faecium*. No resistance to gentamicin, linezolid, teicoplanin, tigecycline or vancomycin was observed in layers in 2023 (see Figure 38).

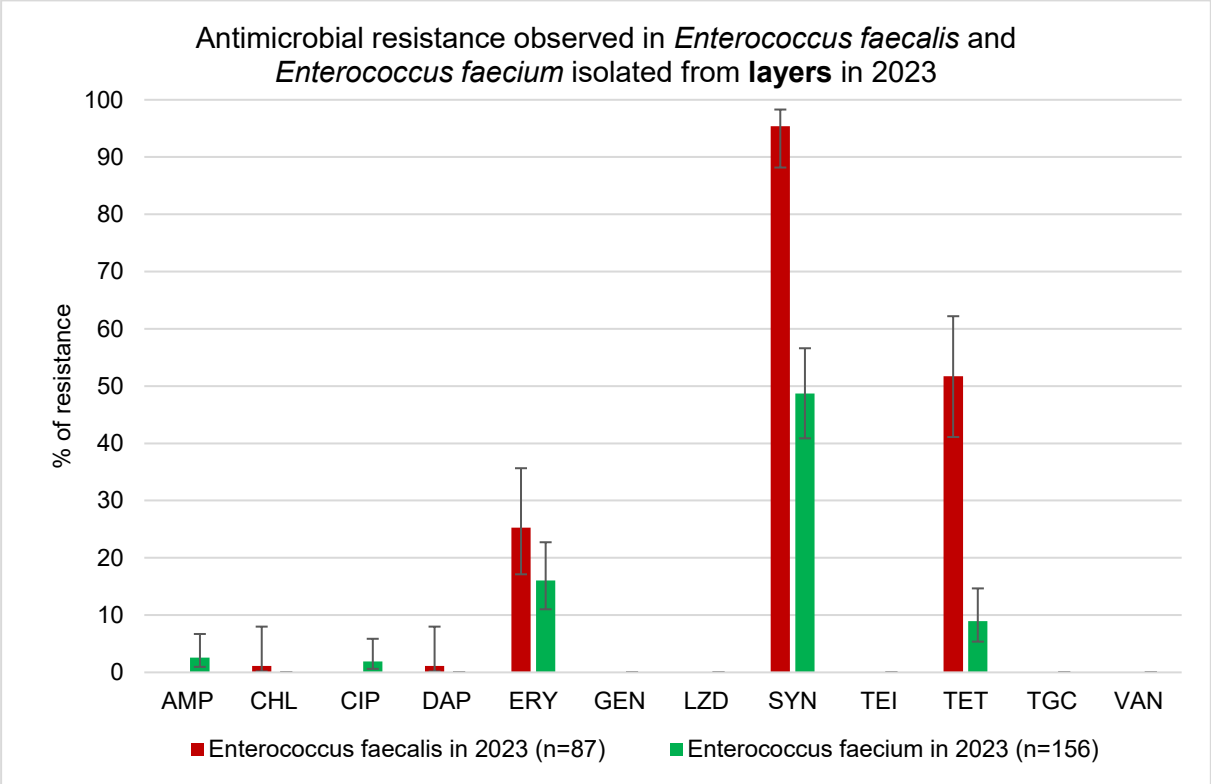

Figure 38. Percentages of antimicrobial resistance in *Enterococcus faecalis* (n=87) and *Enterococcus faecium* (n=156) isolated from layers in 2023.

Based on epidemiological cut-offs according to the European Committee on Antimicrobial Susceptibility (EUCAST) for the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC) and vancomycin (VAN).

3.2.10.3. Antimicrobial resistance observed in *Enterococcus faecium* and *Enterococcus faecalis* isolated from veal calve samples

A total of 228 antimicrobial susceptibility tests were performed after isolation of *Enterococcus faecalis* (n=90) and *Enterococcus faecium* (n=138) from veal calve samples.

Within veal calve samples, extremely high resistance rates were observed for tetracycline (91.1%) and erythromycin (83.3%) in *E. faecalis*. Extremely high resistance rates to quinupristin/dalfopristin (82.6%), and a very high rate to tetracycline resistance (54.3%) and a high level to erythromycin resistance (47.8%) were observed in *E. faecium*. Contrary to a high resistance rate observed in *E. faecalis* (55.6%), resistance to chloramphenicol was low (2.2%) in *E. faecium*.

A moderate rate of resistance to gentamicin (17.8%) was observed in *E. faecalis* while this resistance was very low in *E. faecium* (0.7%). The resistance rate observed to ciprofloxacin was low in *E. faecalis* (2.2%). The resistance rate to ampicillin observed in *E. faecium* was moderate (12.3%).

In total, 12 strains (11 *E. faecalis* and 1 *E. faecium*) showed resistance to linezolid, characterized by a minimum inhibitory concentration of 8 mg/L (n=11) or 16 mg/L (n=1 *E. faecium*). No resistance to daptomycin, teicoplanin, tigecycline or vancomycin was observed in veal calves in 2023 (see Figure 39).

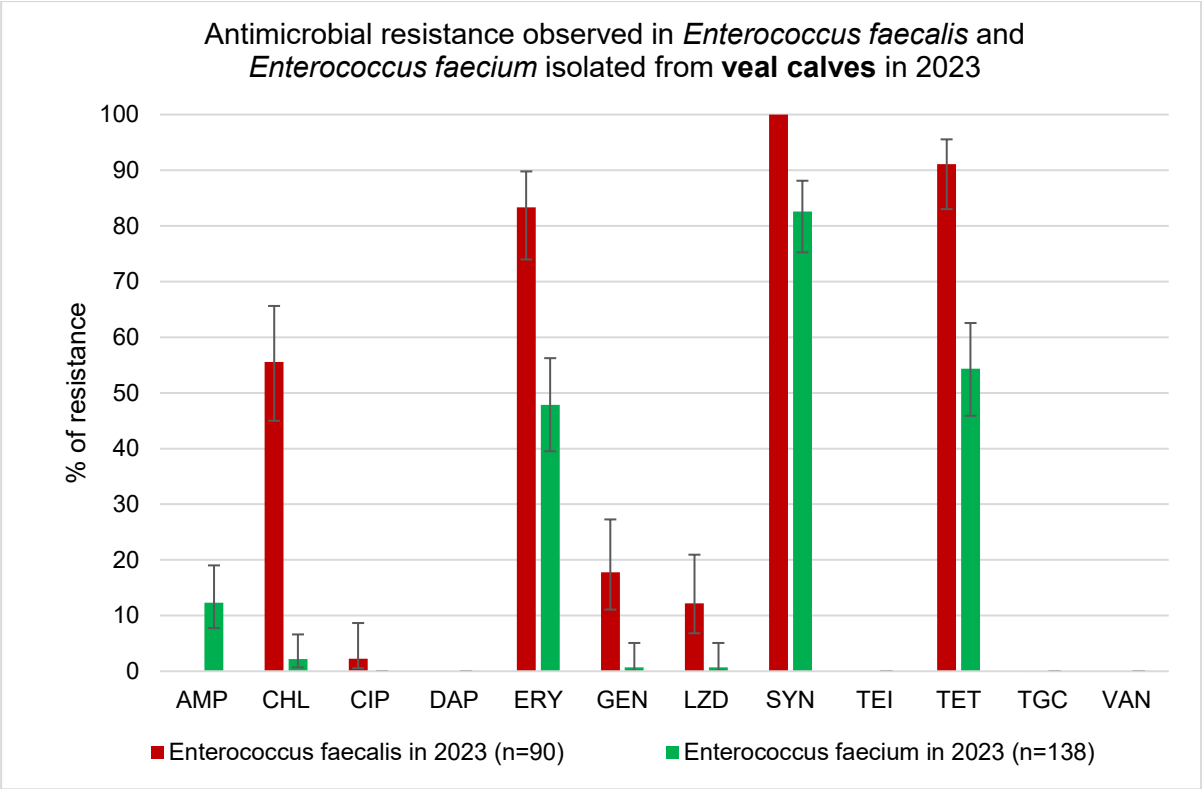

Figure 39. Percentages of antimicrobial resistance in *Enterococcus faecalis* (n=90) and *Enterococcus faecium* (n=138) isolated from veal calves in 2023.

Based on epidemiological cut-offs according to the European Committee on Antimicrobial Susceptibility (EUCAST) for the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC) and vancomycin (VAN).

3.2.10.4. Antimicrobial resistance observed in *Enterococcus faecium* and *Enterococcus faecalis* isolated from pigs samples

A total of 223 antimicrobial susceptibility tests were performed after isolation of *Enterococcus faecalis* (n=56) and *Enterococcus faecium* (n=167) from pig samples.

Within pig samples, a very high rate of resistance to tetracycline (55.4%), and a high rate of resistance to erythromycin (41.1%) were observed in *E. faecalis*. In *E. faecium*, an extremely high percentage of resistance to quinupristin/dalfopristin (79.6%) and high resistance to tetracycline (41.3%) were observed, while the resistance rate to erythromycin was moderate (10.8%). The observed resistance rate to chloramphenicol was moderate in *E. faecalis* (17.9%) while low (1.8%) in *E. faecium*. A low resistance rate to ampicillin (6.6%) was observed in *E. faecium* and no resistance was observed in *E. faecalis*. In addition, a low rate of resistance to gentamicin (8.9%) was observed in *E. faecalis* whereas this resistance was very low (0.6%) in *E. faecium*. Finally, resistance to ciprofloxacin was found in low rates in *E. faecalis* and *E. faecium* (1.8% and 3.0% respectively).

The resistance to daptomycin was observed in a low rate (1.8%) in in *E. faecalis* in 2023. In addition, 4 strains isolated from pig samples (n=3 *E. faecium* and 1 *E. faecalis*) showed resistance to linezolid, characterized by a minimum inhibitory concentration of 8 mg/L (n=4). No resistance to teicoplanin, tigecycline or vancomycin was observed in pigs in 2023 (see Figure 40).

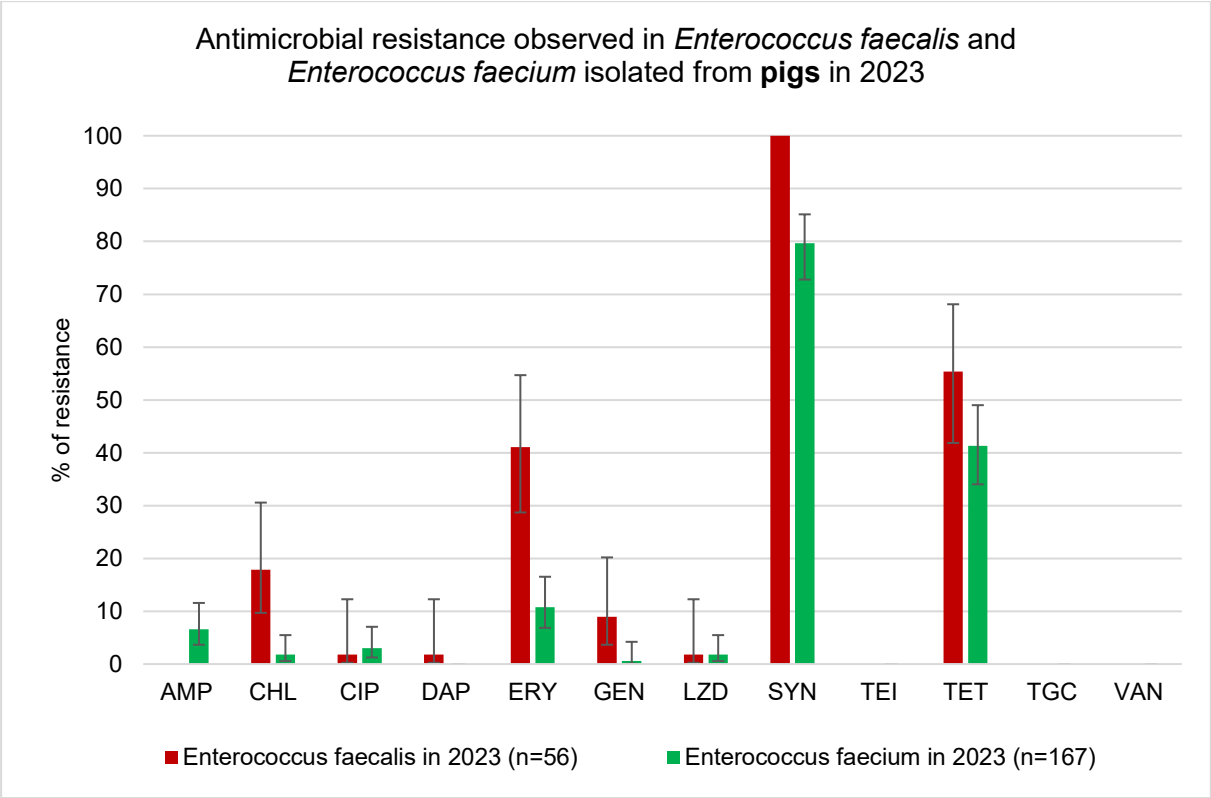

Figure 40. Percentages of antimicrobial resistance in *Enterococcus faecalis* (N=56) and *Enterococcus faecium* (N=167) isolated from pigs in 2023.

Based on epidemiological cut-offs according to the European Committee on Antimicrobial Susceptibility (EUCAST) for the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC) and vancomycin (VAN).

3.2.10.5. Comparison of antimicrobial resistances observed in *Enterococcus faecalis* and *Enterococcus faecium* per animal matrix between 2019 and 2023

In general, the resistance rates observed in *Enterococcus faecalis* and *Enterococcus faecium* isolated from the different animal matrices studied seemed stable since 2019 (see Figure 41 to Figure 45), with the exception of significant decreases in the rate of resistance to certain antimicrobials observed in 2023. Indeed, a significant decrease in erythromycin resistance rate was observed in *E. faecalis* isolated from breeders (-22.7%) in 2023 compared to 2021. A significant decrease of tetracycline resistance (-9.2%) was also observed in *E. faecalis* isolated from broilers in 2023, in comparison to 2021. In 2023, the resistance rate to daptomycin was low in *E. faecalis* isolated from breeders (1.9%), layers (1.1%) and pigs (1.8%), and this resistance was absent in *E. faecium* from all animal categories. This decrease correlates with the modification of the threshold of this resistance within this species (from 4 to 8 mg/L) in 2021. No resistance to teicoplanin, tigecycline or vancomycin was observed in 2023.

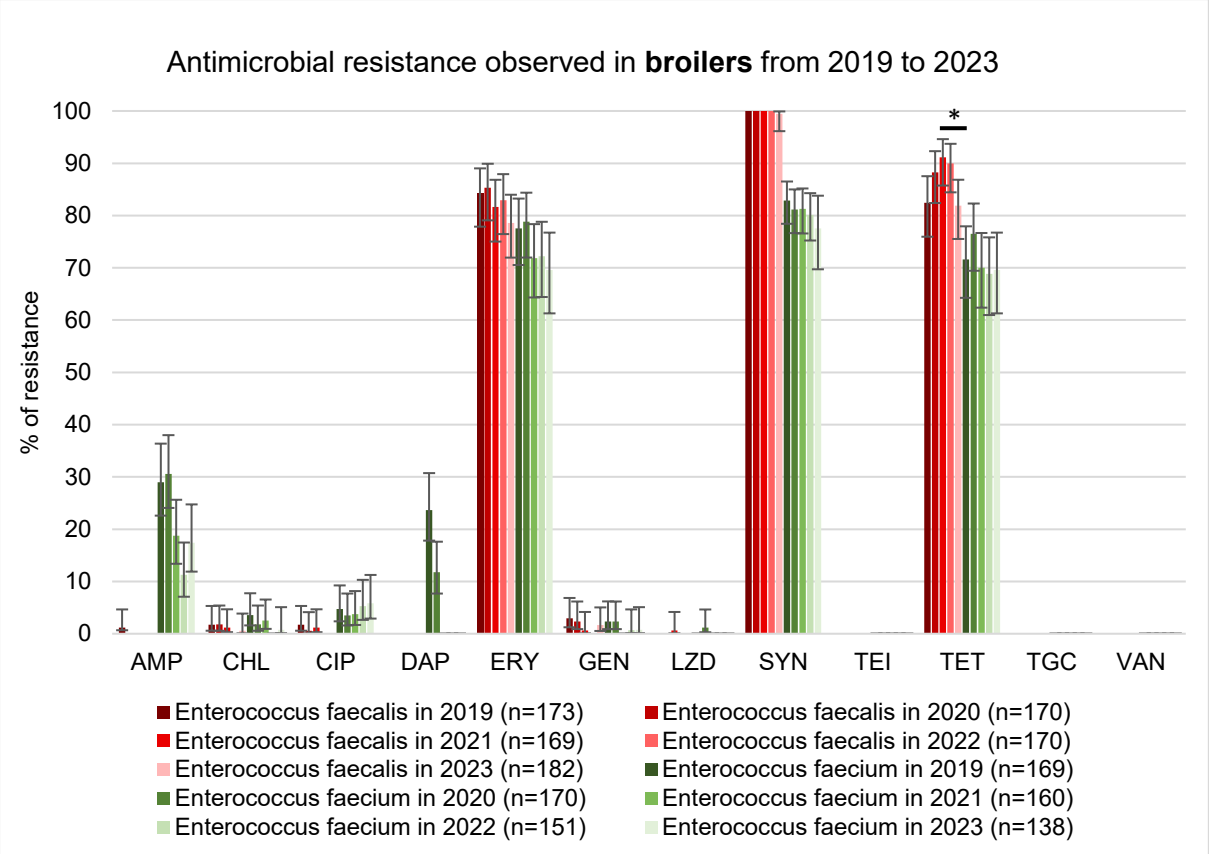

Figure 41. Percentages of antimicrobial resistance in *Enterococcus faecalis* and *Enterococcus faecium* isolated from broilers from 2019 to 2023.

Based on epidemiological cut-offs according to the European Committee on Antimicrobial Susceptibility (EUCAST) for the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC) and vancomycin (VAN). *p value $_{(2021\text{-}2023)}$ <0.05.

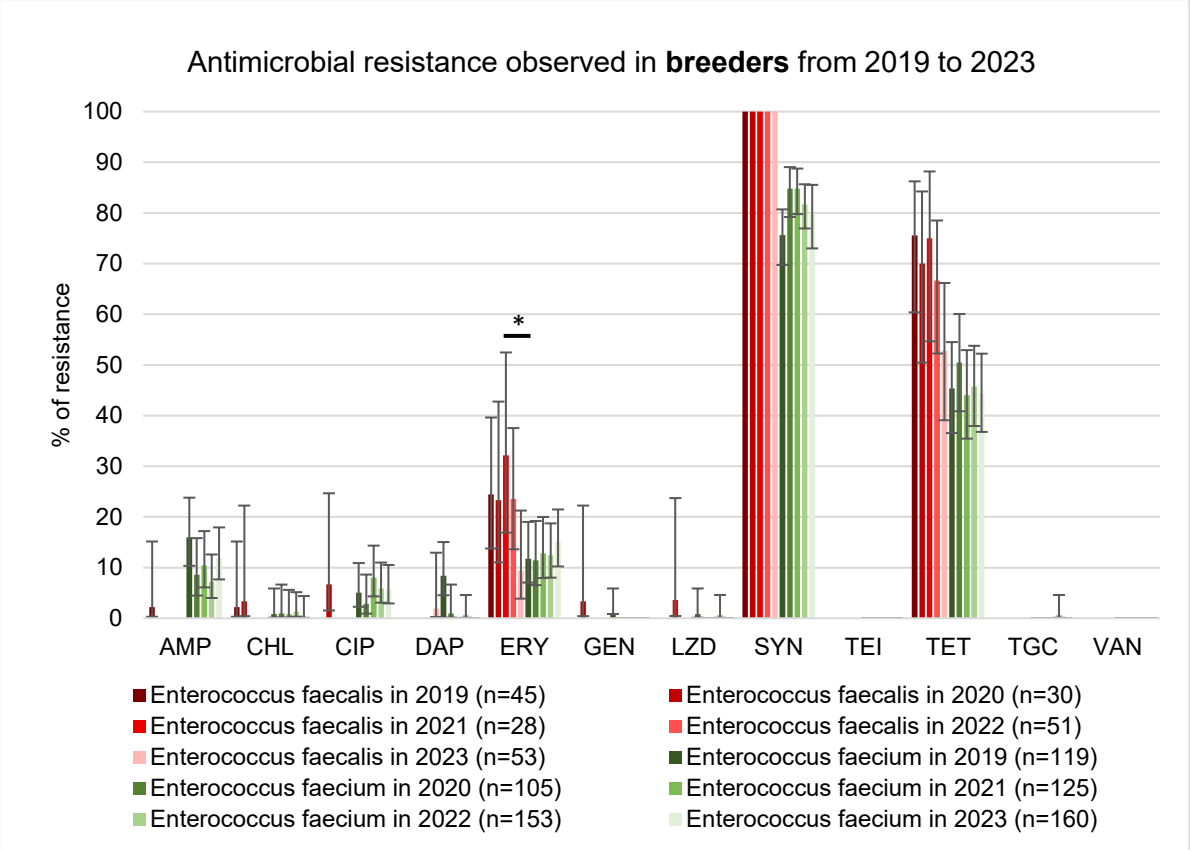

Figure 42. Percentages of antimicrobial resistance in *Enterococcus faecalis* and *Enterococcus faecium* isolated from breeders from 2019 to 2023.

Based on epidemiological cut-offs according to the European Committee on Antimicrobial Susceptibility (EUCAST) for the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC) and vancomycin (VAN). *p value $_{(2021\text{-}2023)}$ <0.05.

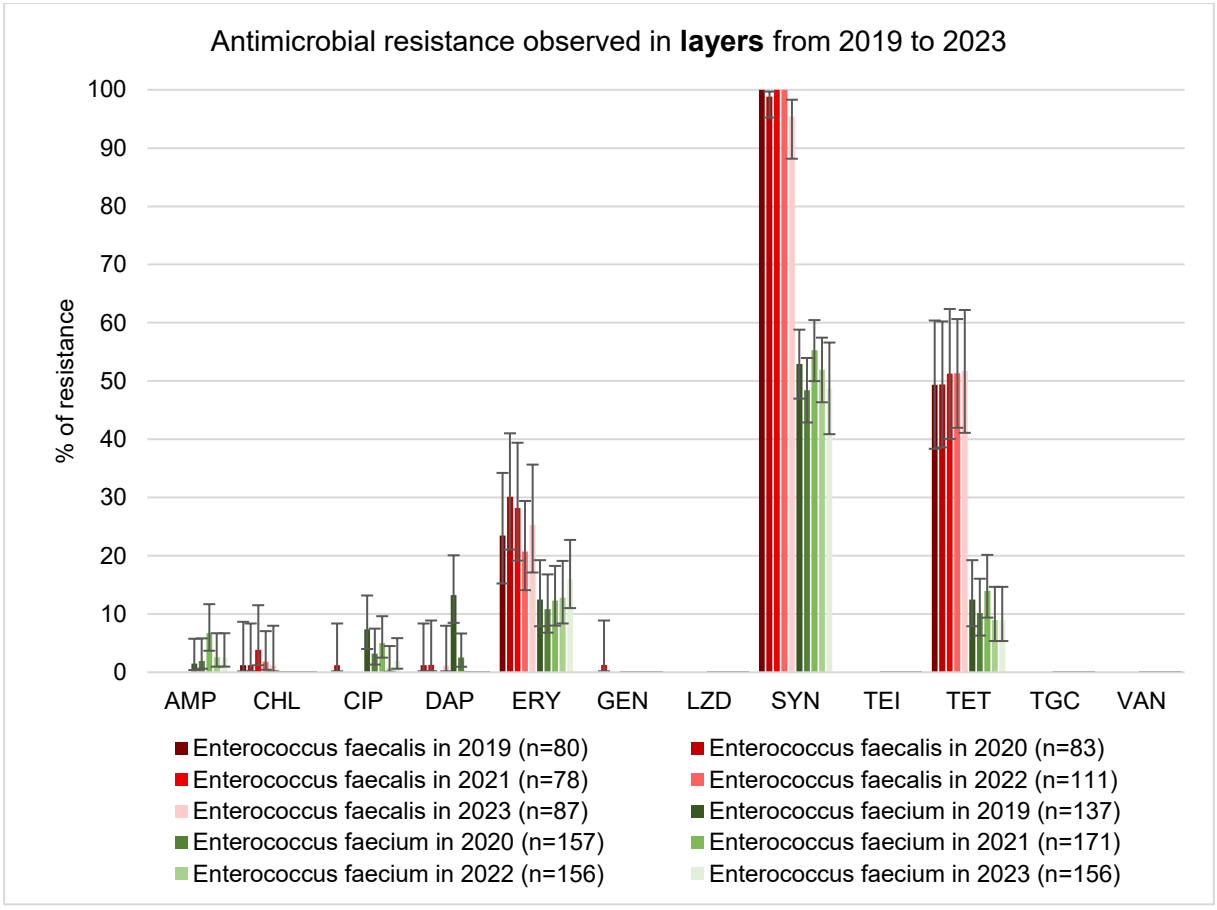

Figure 43. Percentages of antimicrobial resistance in *Enterococcus faecalis* and *Enterococcus faecium* isolated from layers from 2019 to 2023.

Based on epidemiological cut-offs according to the European Committee on Antimicrobial Susceptibility (EUCAST) for the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC) and vancomycin (VAN).

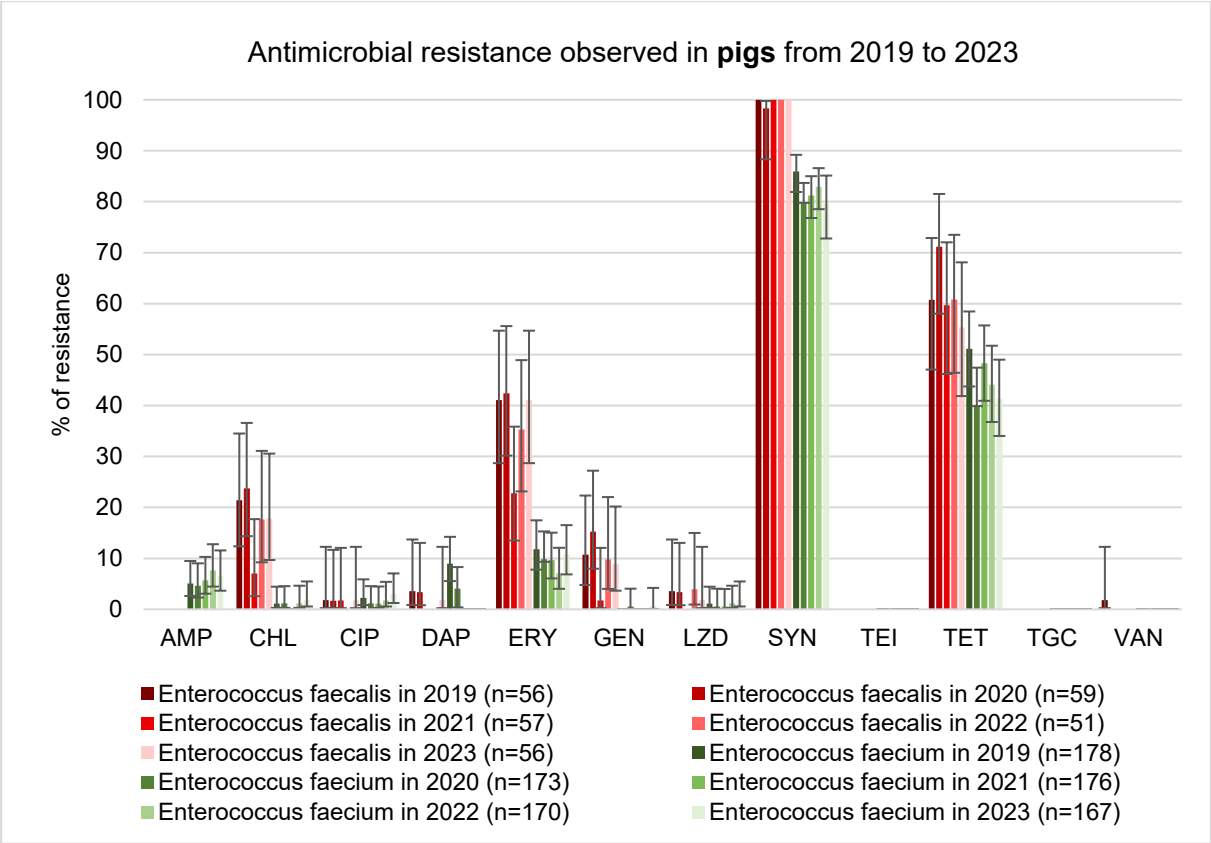

Figure 44. Percentages of antimicrobial resistance in *Enterococcus faecalis* and *Enterococcus faecium* isolated from pigs from 2019 to 2023.

Based on epidemiological cut-offs according to the European Committee on Antimicrobial Susceptibility (EUCAST) for the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC) and vancomycin (VAN).

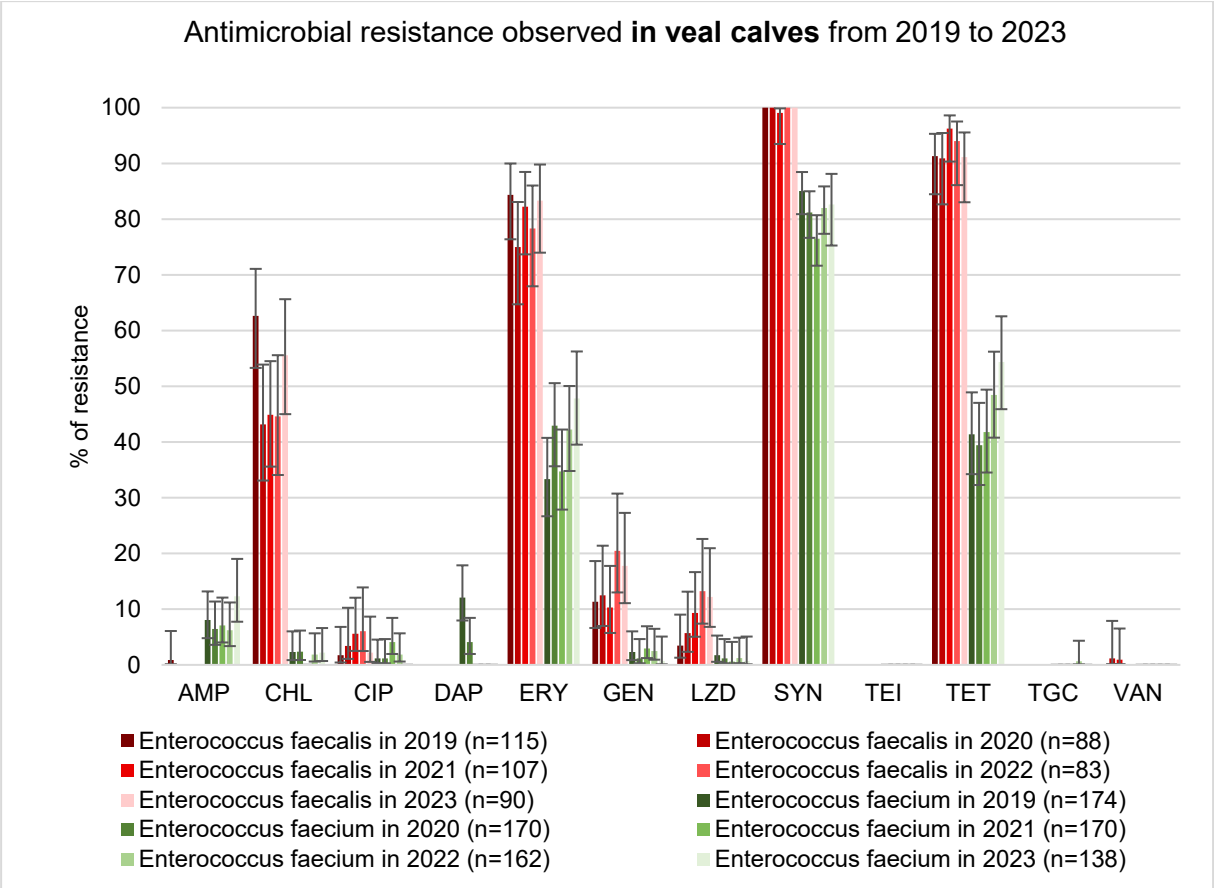

Figure 45. Percentages of antimicrobial resistance in *Enterococcus faecalis* and *Enterococcus faecium* isolated from veal calves from 2019 to 2023.

Based on epidemiological cut-offs according to the European Committee on Antimicrobial Susceptibility (EUCAST) for the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC) and vancomycin (VAN).

3.2.10.6. Multiresistance observed in *Enterococcus faecalis* and *Enterococcus faecium* per animal matrix

It should be noted that resistance to quinupristin/dalfopristin, intrinsic in *E. faecalis*, was counted only in *Enterococcus faecium* for the calculation of multi-resistance and the comparison of the resistance profiles observed between *Enterococcus faecalis* and *Enterococcus faecium*.

In 2023, no significant variation in rates of multi-resistance was observed among enterococci. The highest number of multi-resistant strains was observed in veal calves (63.3% of *E. faecalis*) and in broilers (53.6% of *E. faecium*) (see Figure 47 and Figure 49). *E. faecalis* isolated from broilers were mostly resistant to 1 (17.0%) or 2 (70.9%) antimicrobials, while 10.9%, 24.6%, 34.1%, 18.8% and 0.7% of *E. faecium* were resistant to 1, 2, 3, 4 or 5 antimicrobials respectively. Among poultry, the strains isolated from breeding hens and laying hens were globally less resistant. Indeed, 49.1% and 34.5% of *E. faecalis* and 36.3% and 40.4% of *E. faecium* resistant to 1 antimicrobial as well as 7.5% and 20.7% of *E. faecalis* and 35.6% and 10.3% of *E. faecium* resistant to 2 different antimicrobials, respectively. In addition, 1.1% of *E. faecalis* isolated from layers, and 13.1% and 5.8% of *E. faecium* isolated from breeders and layers respectively, were resistant to 3 antimicrobials. In breeders, 1.9% and 0.6% of *E. faecium* were also resistant to 4 and 5 antimicrobials, respectively. Within pig samples, 28.6% of *E. faecalis* were resistant to 1, 14.3% to 2, 8.9% to 3, 8.9% to 4 and 1.8% to 5 different antimicrobials. Among *E. faecium* isolated from pigs, 38.3% were resistant to 1 antimicrobial, 32.9% to 2, 9.6% to 3, 0.6% to 4, 1.2% to 5 and 0.6% to 7 antimicrobials. In veal calves, *E. faecalis* were resistant to 1 (8.9%),

2 (20.0%), 3 (43.3%), 4 (16.7%) and 5 (3.3%) different antimicrobials. Also, 34.1%, 27.5%, 21.7% and 11.6% of *E. faecium* isolated from veal calves were resistant to 1, 2, 3 and 4 antimicrobials, respectively (see

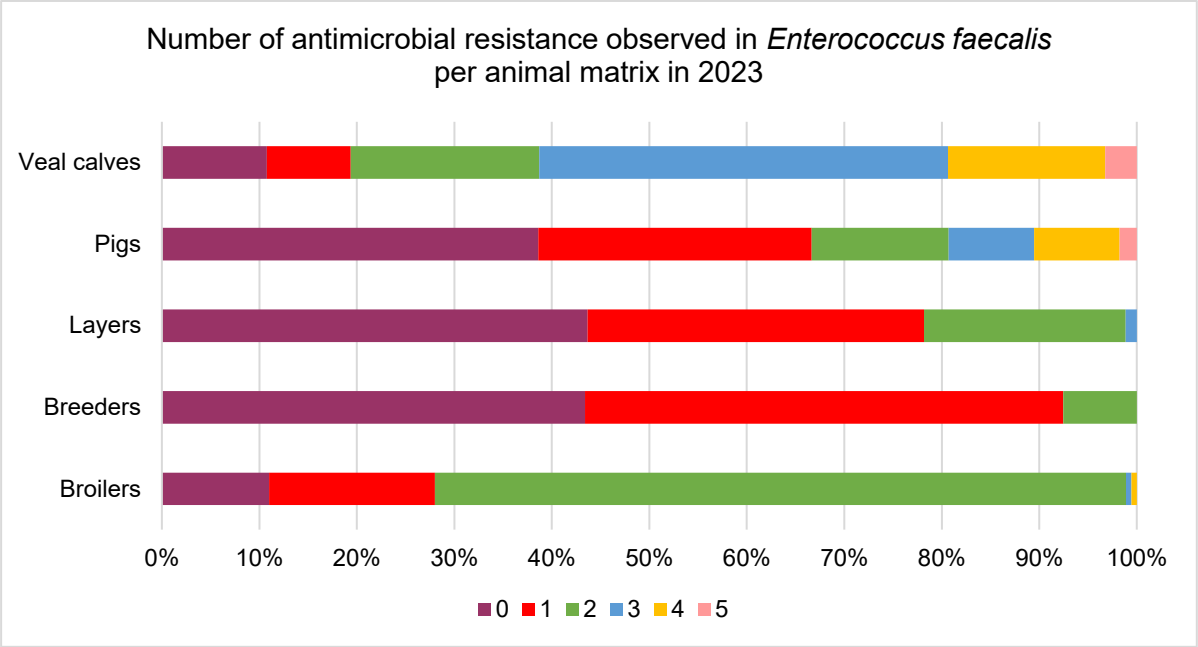

Figure **46**,

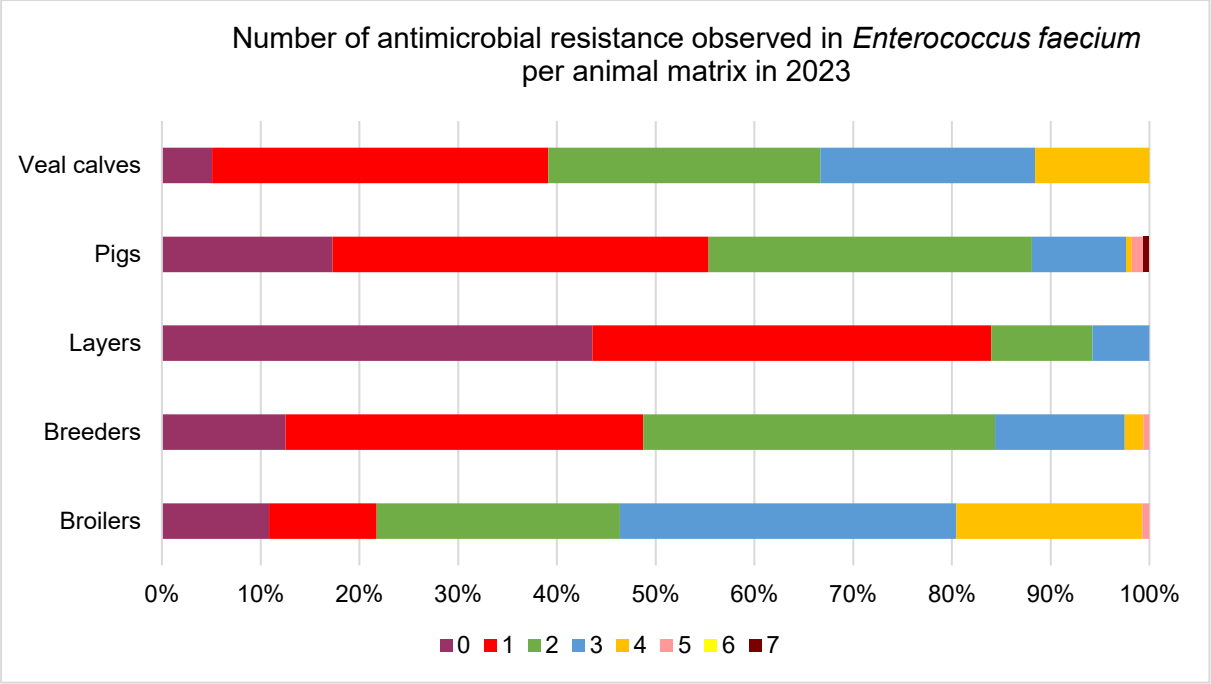

Figure **48**). The phenotypic resistance profiles observed in *E. faecalis* and in *E. faecium* per animal matrix in 2023 are represented in Table 24 and Table 25.

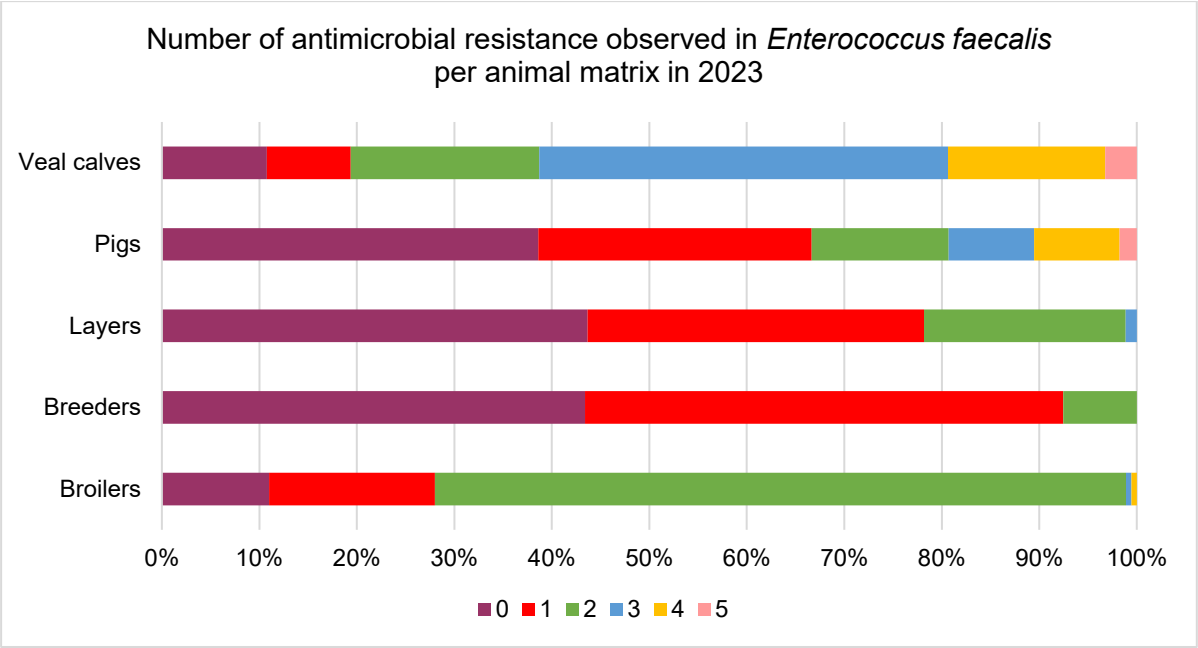

Figure 46. Percentages of *Enterococcus faecalis* according to the number of resistance and the animal matrix in 2023.

The color legend indicates the number of families of antibiotics to which the strains are resistant, the abscissa indicates the percentage of strains resistant to the corresponding number of antibiotics. Resistance to quinupristin/dalfopristin in *Enterococcus faecalis* was not taken into account in the multidrug resistance calculation.

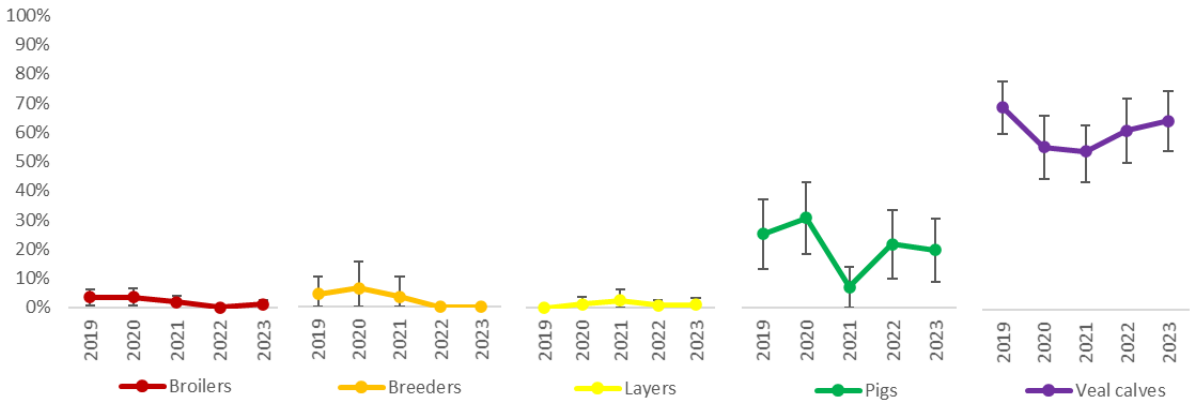

Figure 47. Percentages of MDR *Enterococcus faecalis* observed per animal matrix between 2019 and 2023.

A strain is considered multi-resistant when it is resistant to at least 3 different families of antibiotics. Resistance to quinupristin/dalfopristin in *Enterococcus faecalis* was not taken into account in the multidrug resistance calculation.

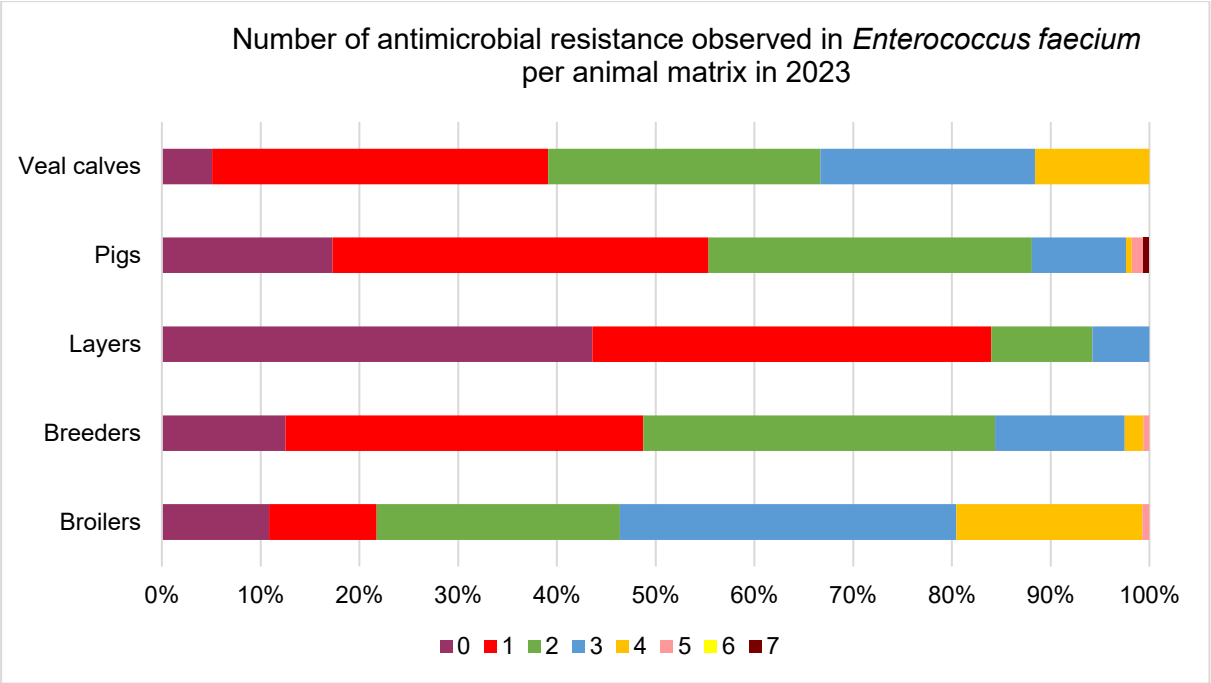

Figure 48. Percentages of *Enterococcus faecium* according to the number of resistance and the animal matrix in 2023.

The color legend indicates the number of families of antibiotics to which the strains are resistant, the abscissa indicates the percentage of strains resistant to the corresponding number of antibiotics.

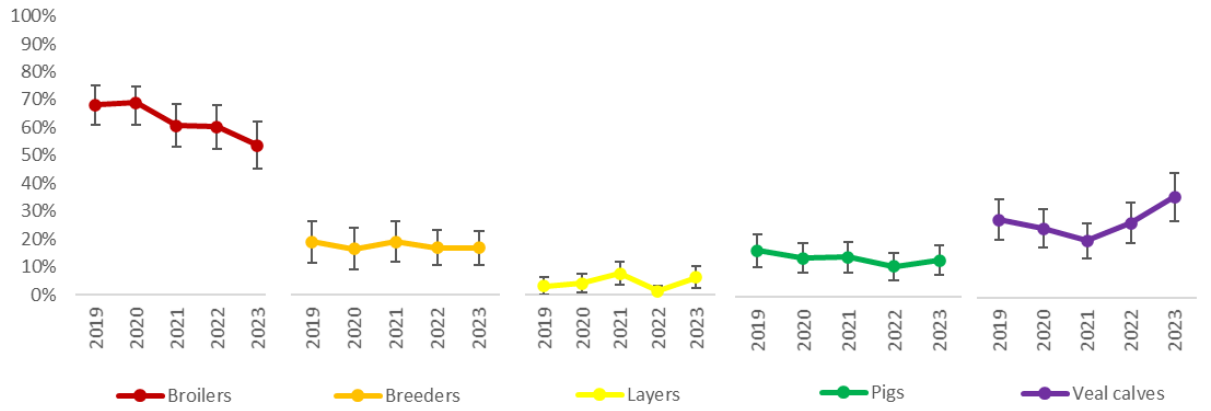

Figure 49. Percentages of MDR *Enterococcus faecium* observed per animal matrix between 2019 and 2023.

A strain is considered multi-resistant when it is resistant to at least 3 different families of antibiotics.

MDR	Resistance profiles	Broilers (n=182)	Breeders (n=53)	Layers (n=87)	Pigs (n=56)	Veal calves (n=90)
5	CHL ERY GEN LZD TET	-	-	-	-	1
	CHL CIP ERY GEN TET					1
	CHL CIP ERY LZD TET	-	-	-	1	1
4	CHL ERY GEN TET	1	-	-	4	8
	CHL ERY LZD TET					6
	ERY GEN LZD TET	-	-	-		1
	CHL DAP ERY TET	-	-	-	1	-
3	CHL ERY TET	-	-	1	4	32
	ERY GEN TET	1	-		1	5
	ERY LZD TET	-	-			2
2	CHL ERY	-	-	-		1
	ERY GEN	1	-		-	-
	ERY TET	128	4	18	8	17
1	DAP		1	1		
	ERY	12	1	3	4	
	TET	19	24	26	12	8

Table 24. List of phenotypic resistance profiles observed in *Enterococcus faecalis* isolates and classified by animal matrix in 2023.

MDR	Resistance profiles	Broilers (n=182)	Breeders (n=53)	Layers (n=87)	Pigs (n=56)	Veal calves (n=90)
5	CHL ERY GEN LZD TET	-	-	-	-	1
	CHL CIP ERY GEN TET	-	-	-	-	1
	CHL CIP ERY LZD TET	-	-	-	1	1
4	CHL ERY GEN TET	1	-	-	1	8
	CHL ERY LZD TET	-	-	-	-	6
	ERY GEN LZD TET	-	-	-	-	1
	CHL DAP ERY TET	-	-	-	4	-
3	CHL ERY TET	-	-	1	4	32
	ERY GEN TET	1	-	-	1	5
	ERY LZD TET	-	-	-	-	2
2	CHL ERY	-	-	-	-	1
	ERY GEN	1	-	-	-	-
	ERY TET	128	4	18	8	17
1	DAP	-	1	1	-	-
	ERY	12	1	3	4	-
	TET	19	24	26	12	8

The MDR “Multi-Drug resistance” column corresponds to multi-resistance, i.e., the number of resistances observed to each family of antimicrobials. The number N corresponds to the number of strains presenting the resistance profile. Resistance to quinupristin/dalfopristin was not taken into account in the table of resistance profiles observed in *E. faecalis*.

Table 25. List of phenotypic resistance profiles observed in *Enterococcus faecium* isolates and classified by animal matrix in 2023.

MDR	Resistance profiles	Broilers (n=138)	Breeders (n=160)	Layers (n=156)	Pigs (n=167)	Veal calves (n=138)
7	AMP CHL ERY GEN LZD SYN TET	-	-	-	1	-
5	AMP CIP ERY SYN TET	1	1	-	-	-
	CHL ERY LZD SYN TET	-	-	-	2	-
4	AMP ERY SYN TET	22	2	-	1	12
	CHL ERY SYN TET	-	1	-	-	2
	CHL ERY LZD TET	-	-	-	-	1
	CIP ERY SYN TET	3	-	-	-	-
	ERY GEN SYN TET	1	-	-	-	1
3	AMP ERY SYN	1	-	-	-	-
	AMP SYN TET	-	7	1	5	4
	AMP CIP SYN	-	-	1	-	-
	CIP ERY SYN	1	-	-	-	-
	CIP SYN TET	-	4	-	2	-
	CHL ERY TET	1	-	-	-	-
	ERY SYN TET	44	10	7	9	26
2	AMP SYN	-	9	1	2	1
	AMP ERY	-	-	1	-	-
	CIP SYN	2	4	1	3	-
	ERY SYN	10	8	10	5	12
	ERY TET	10	1	2	-	11
	SYN TET	12	35	1	45	14
1	AMP	-	-	-	2	-
	CIP	1	-	1	-	-
	ERY	2	1	5	-	1
	SYN	10	47	54	58	42
	TET	2	10	3	4	4

The MDR "Multi-Drug resistance" column corresponds to multi-resistance, i.e., the number of resistances observed to each family of antimicrobials. The number N corresponds to the number of strains presenting the resistance profile.

3.2.10.7. Investigation by WGS of *Enterococcus faecalis* and *Enterococcus faecium* isolated in 2023

- Selection criteria

In 2023, 19 enterococci (15 *E. faecalis* and 4 *E. faecium*) isolated from pigs (n=5), breeders (n=1), layers (n=1) and veal calves (n=12) were investigated by sequencing. Each strain was selected according to its phenotypic profile, independently of the animal matrix and presented the following criteria: resistance to linezolid or resistance to daptomycin. No phenotypic resistance to vancomycin was observed in *Enterococcus* spp. in 2023.

- MLST typing of enterococci

By sequencing, typing of enterococci by Multi Locus Sequence Typing (MLST) allowed to identify 13 different sequence-types (STs), of which 11 STs were observed only once, 1 STs (ST 40) observed in 3 different strains (n=2 veal calves, n=1 layers), and 1 STs (ST 21) observed in 5 strains (isolated from veal calves and pigs); and some of which had the same phenotype (see Table 26). In order to determine whether the strains presenting the same ST were genetically linked, their cgMLST ("core genome MLST") profiles were compared, based on different thresholds as following : 0 alleles=definitely related,

1-5 alleles=very likely related, 6-10 alleles=likely related and >25 alleles=not likely related. This comparison highlighted a genetic similarity observed between two of these strains.
Indeed, the comparison of the 2 *E. faecalis* (VAR 1090 and VAR 1129) isolated from veal calves characterized by an ST21 with a same resistance phenotype (see Table 26) showed that these strains were likely related (with 8 allelic differences observed).

Table 26. List of enterococci sequenced by WGS in 2023 and their phenotypic profile obtained from the antimicrobial susceptibility study.

Sciensano VAR ID	Species	Phenotype	ST	Animal matrix
VAR-1155	*E. faecalis*	DAP	40	Layers
VAR-1156	*E. faecalis*	DAP	118	Breeders
VAR-1157	*E. faecalis*	CHL DAP ERY TET	58	Pigs
VAR-1090	*E. faecalis*	CHL ERY LZD TET	21	Veal calves
VAR-1092	*E. faecalis*	CHL ERY LZD TET	21	Veal calves
VAR-1129	*E. faecalis*	CHL ERY LZD TET	21	Veal calves
VAR-1130	*E. faecalis*	CHL ERY LZD TET	21	Veal calves
VAR-1104	*E. faecalis*	CHL ERY LZD TET	23	Veal calves
VAR-1081	*E. faecalis*	CHL ERY LZD TET	40	Veal calves
VAR-1083	*E. faecalis*	CHL ERY LZD TET	41	Veal calves
VAR-1128	*E. faecalis*	CHL ERY LZD TET	314	Veal calves
VAR-1159	*E. faecalis*	ERY GEN LZD TET	25	Veal calves
VAR-1127	*E. faecalis*	CHL CIP ERY LZD TET	330	Veal calves
VAR-1093	*E. faecalis*	CHL CIP ERY LZD TET	1022	Pigs
VAR-1094	*E. faecalis*	CHL ERY GEN LZD TET	40	Veal calves
VAR-1082	*E. faecium*	CHL ERY LZD TET	2208	Veal calves
VAR-1091	*E. faecium*	CHL ERY LZD SYN TET	21	Pigs
VAR 1147	*E. faecium*	CHL ERY LZD SYN TET	324	Pigs
VAR-1132	*E. faecium*	AMP CHL ERY GEN LZD SYN TET	184	Pigs

VAR-ID : internal number for WGS analysis. Phenotypes interpreted based on European Antimicrobial Susceptibility Committee (EUCAST) epidemiological cut-offs for the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN), teicoplanin (TEI), tetracycline (TET), tigecycline (TGC) and vancomycin (VAN). The strains were classified according to the following characteristics: 1) bacterial species, 2) phenotype, 3) ST and 4) matrix.

- Genotyping of observed antimicrobial resistance

Sequencing of enterococci revealed the presence of resistance genes explaining the resistant phenotype in 92.6% (n=87/94) of individual observed resistance phenotypes (see Table 27).
All linezolid-resistant strains analyzed (n=16) were characterized by the presence of the *optrA* gene (n=15 with 12 isolated from veal calves and 3 isolated from pigs) or the combination of *optrA*/*poxtA* (n=1 isolated from pigs). Resistance to erythromycin (n=17) was characterized by the presence of *erm(A)* (n=1), *erm(B)* (n=3), *erm(A)/erm(B)* (n=9), *erm(A)/msr(C)* (n=2), *erm(B)/msr(C)* (n=1) and *erm(A)/erm(B)/msr(C)* (n=1). Of 17 strains resistant to chloramphenicol, all carried at least one resistance gene, namely *fexA* (n=10), *fexB* (n=1), *cat* (n=1), *cat/fexA* (n=2) and $catp_{C221}$/*fexA* (n=3). Resistance to quinupristin/dalfopristin (Q/D, SYN) was characterized by the presence of the *lsaA* (n=13) or *lsaA/lsaE* (n=2) genes in *E. faecalis* (n=15) and by the presence of *msr(C)* (n=1) or *lsa(E)/msr(C)* (n=2) in *E. faecium* (n=3). The *msrC* gene was also found in one Q/D-susceptible *E. faecium* but resistant to erythromycin, supporting this gene is probably involved in resistance to several antibiotics, i.e., macrolides and streptogramins, as already described elsewhere (Smoglica *et al.*, 2022, Zaheer *et*

al., 2020). The *aac(6')-aph(2")* gene known to confer resistance to gentamicin was found in 3 out of 3 of the resistant strains investigated by WGS. Except one *E. faecalis*, all tetracycline resistant strains sequenced (n=17) carried at least one resistance gene, namely *tet(M)* (n=5) or the combination of 2 genes *tet(L)/tet(M)* (n=11). The ampicillin resistance observed in one sequenced *E. faecium* was explained by the presence of several mutations in the *pbp5* gene, which increase its production (see Table 27). Resistance to ciprofloxacin, and to fluoroquinolones in general, is characterized by the presence of mutations in the *parC* and *gyrA* genes. Mutations were observed in our 2 resistant strains. More specifically, each strain had at least one mutation in *parC* or *gyrA*, with the following observed mutations: *parC* p.S80I (n=2, described in *Enterococcus* spp. (Lopez *et al.,* 2011)), *gyrA* p.S83I (n=1), *gyrA* p.E87G (n=1) and *gyrA* p.D759N (n=1). In 2023, daptomycin resistance was observed in 3 *E. faecalis* (1 isolated from breeder, 1 from layer and 1 from pig). In enterococci, resistance to daptomycin is likely to be associated with mutations in intrinsic genes, such as *liaFSR, cls* or *gdpD* (Bender *et al*, 2018). However, mechanisms leading to this resistance are not yet well understood, making them difficult to target.

In addition to antimicrobial resistance genes, no gene conferring resistance to disinfectants was detected in enterococci in 2023.

Note that most of these genes can be detected with a bead-array developed by the NRL which has been published in MicrobiologyOpen (Kowalewicz *et al.*, 2022).

Table 27. List of resistance genes or resistance mutations identified by WGS per resistant phenotype observed in enterococci in 2023.

Sciensano ID	Species	Animal matrix	AMP	CHL	CIP	DAP	ERY	GEN	LZD	SYN	TET
VAR-1081	*E. faecalis*	Veal calves		*fexA*			*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*
VAR-1083	*E. faecalis*	Veal calves		*cat(p_{C221}), fexA*			*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(M)*
VAR-1090	*E. faecalis*	Veal calves		*fexA*			*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*
VAR-1092	*E. faecalis*	Veal calves		*cat(p_{C221}), fexA*			*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(M)*
VAR-1093	*E. faecalis*	Pigs		*cat, fexA*	*gyrA p.S83I, gyrA p.D759N, parC p.S80I*		*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*
VAR-1094	*E. faecalis*	Veal calves		*fexA*			*erm(A)*	*aac(6')-aph(2'')*	*optrA*	*lsa(A), lsa(E)*	*tet(L), tet(M)*
VAR-1104	*E. faecalis*	Veal calves		*fexA*			*erm(A), erm(B)*		*optrA*	*lsa(A)*	-
VAR-1127	*E. faecalis*	Veal calves		*cat, fexA*	*gyrA p.E87G, parC p.S80I*		*erm(B)**		*optrA*	*lsa(A)*	*tet(L), tet(M)*
VAR-1128	*E. faecalis*	Veal calves		*fexA*			*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*
VAR-1129	*E. faecalis*	Veal calves		*cat(p_{C221}), fexA*			*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*
VAR-1130	*E. faecalis*	Veal calves		*fexA*			*erm(B)*		*optrA*	*lsa(A), lsa(E)*	*tet(L), tet(M)*
VAR 1159	*E. faecalis*	Veal calves		*fexA*			*erm(A), erm(B)*	*aac(6')-aph(2'')*	*optrA*	*lsa(A)*	*tet(L), tet(M)*
VAR 1155	*E. faecalis*	Layers				-				*lsa(A)*	
VAR 1156	*E. faecalis*	Breeders				-				*lsa(A)*	
VAR 1157	*E. faecalis*	Pigs		*cat*		-	*erm(B)*			*lsa(A)*	*tet(L), tet(M)*
VAR-1082	*E. faecium*	Veal calves		*fexA*			*erm(A), msr(C)*		*optrA*	*msr(C)*	*tet(M)*
VAR-1091	*E. faecium*	Pigs		*fexB*			*erm(A), erm(B), msr(C)*		*optrA, poxtA*	*lsa(E), msr(C)*	*tet(L), tet(M)*
VAR 1147	*E. faecium*	Pigs		*fexA*			*erm(A), msr(C)*		*optrA*	*msr(C)*	*tet(M)*
VAR-1158	*E. faecium*	Pigs	*pbp5 p.V24A, p.S27G, p.R34Q, p.G66E, p.E100Q, p.K144Q, p.T172A, p.L177I, p.A216S, p.T324A, p.N496K, p.A499I, p.E525D*	*fexA*			*erm(B), msr(C)*	*aac(6')-aph(2'')*	*optrA*	*lsa(E), msr(C)*	*tet(M)*

VAR-ID : internal number for WGS analysis. Each orange box corresponds to the presence of phenotypic resistance to the antimicrobial cited, with the following antimicrobials: ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), daptomycin (DAP), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN) and tetracycline (TET). Each gene or mutation found by WGS is indicated below each antimicrobial to which it confers resistance. The presence of a "-" dash indicates a resistant phenotype for which no resistance gene or mutation was detected by WGS.

*A percentage of coverage <100% was observed for the *erm(B)* gene, i.e., the *erm(B*) gene length in this strain was shorter than the reference gene length (alignment length : 567/768).

- Virulence genes

Generally speaking, virulence is a common trait of *Enterococcus* spp. regardless of their origin (animal, human, environmental or food) allowing them to adapt to their environment. The different virulence factors identified within enterococci ensure different functions such as adhesion to a host or to a non-biological environment, conjugation/formation of the pili, formation of a protective biofilm or cytolytic activity allowing colonization. or nutrient supply (Selleck *et al*., 2019; Soheili *et al*., 2014; Semedo *et al*., 2003). In addition, the presence of certain virulence factors, although their precise role in pathogenicity is not fully known, has been particularly associated with *Enterococcus faecalis* isolated from infections (Mannu *et al.,* 2003). Indeed, virulence factors such as *agg*, *esp* or *cyl* located on islands of pathogenicity, have been associated with greater virulence due to their function (aggregation, biofilm formation, cytolytic) and the higher frequency of their presence in clinical strains (Selleck *et al*., 2019, Rathnayake *et al*., 2012; Mannu *et al*., 2003).

Analysis of 19 enterococci showed that a greater number of virulence factors were found in *E. faecalis* compared to *E. faecium* (see Table 28), regardless of the animal origin of these strains as described elsewhere (Jimenez *et al*., 2013; Rathnayake *et al*., 2012). All strains investigated by WGS in 2023 carried at least one virulence factor, namely *efaAfs/efaAfm* (role of adhesins) specific for *E. faecalis* and *E. faecium* respectively. Among all the strains analyzed, none was characterized by the presence of the virulence factor *cyl*, known for its cytolytic activity (Selleck *et al*., 2019; Semedo *et al*., 2003). In addition, the adhesin *esp* observed frequently in clinical *E. faecium* strains (Selleck *et al*., 2019; Rathnayake *et al*., 2012; Mannu *et al*., 2003) was neither isolated within strains isolated from food-producing animals in 2023. The aggregation substance protein Agg, which is associated with greater virulence and higher frequency of their presence in clinical strains, was detected in 10 *E. faecalis* on the 19 investigated isolates.

Table 28. List of virulence factors identified by WGS in enterococci in 2023.

VAR ID	Species	efaA	ElrA	StrA	Acm/ Ace	ccf/cob/cad			camE	agg	tpx	ebp			hyl		gelE	fsr
VAR-1081 VAR-1094 VAR-1127 VAR-1130	*E. faecalis*	efaAfs	ElrA	SrtA	ace	cCF10	cOB1	cad	camE	agg	tpx	ebpA	ebpB	ebpC	hylA	hylB	gelE	fsrB
VAR-1128 VAR-1155	*E. faecalis*	efaAfs	ElrA	SrtA	ace	cCF10	cOB1	cad	camE	-	tpx	ebpA	ebpB	ebpC	hylA	hylB	gelE	fsrB
VAR-1090 VAR-1092 VAR-1129	*E. faecalis*	efaAfs	ElrA	SrtA	ace	cCF10	cOB1	cad	camE	agg	tpx	ebpA	ebpB	ebpC	hylA	hylB	gelE	-
VAR-1157	*E. faecalis*	efaAfs	ElrA	SrtA	ace	cCF10	cOB1	cad	camE	-	tpx	ebpA	ebpB	ebpC	hylA	-	gelE	fsrB
VAR-1156	*E. faecalis*	efaAfs	-	SrtA	ace	cCF10	cOB1	cad	camE	-	tpx	ebpA	ebpB	ebpC	-	hylB	gelE	fsrB
VAR-1083 VAR-1159	*E. faecalis*	efaAfs	ElrA	SrtA	ace	cCF10	cOB1	cad	camE	agg	tpx	ebpA	ebpB	ebpC	hylA	-	gelE	-
VAR-1093	*E. faecalis*	efaAfs	ElrA	SrtA	ace	cCF10	cOB1	cad	camE	agg	tpx	ebpA	ebpB	ebpC	hylA	-	-	-
VAR-1104	*E. faecalis*	efaAfs	ElrA	SrtA	ace	cCF10	cOB1	cad	camE	-	tpx	ebpA	ebpB	ebpC	hylA	-	-	-
VAR-1082 VAR-1158	*E. faecium*	efaAfm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VAR-1091 VAR-1147	*E. faecium*	efaAfm	-	-	acm	-	-	-	-	-	-	-	-	-	-	-	-	-

VAR-ID : internal number for WGS analysis.

3.2.10.8. Discussion

During enterococci monitoring in 2023, 1183 MALDI-TOF identification tests were performed from 1185 samples taken from poultry, pigs and veal calves, with the remaining 2 samples showing no growth of presumptive enterococci. *Enterococcus faecium* was more frequently isolated than *Enterococcus faecalis* within the samples of breeding hens (88.4%), laying hens (72.0%), veal calves (54.8%) and pigs (62.3%). Conversely, *E. faecalis* was isolated more frequently than *E. faecium* in broiler (68.8%) samples. The prevalences of bacterial species by animal category observed in 2023 were similar to those observed in previous years. After identification of the bacterial species, 468 antimicrobial susceptibility tests in *Enterococcus faecalis* as well as 759 antimicrobial susceptibility tests in *Enterococcus faecium* were carried out.

In general, the resistance rates observed in *Enterococcus faecalis* and *Enterococcus faecium* within the different animal matrices studied seemed stable since 2019, with the exception of significant decreases in the rate of resistance to certain antimicrobials observed in 2023. In 2023, resistance to tetracycline, erythromycin and quinupristin/dalfopristin were still the most observed resistances, both in *E. faecalis* and *E. faecium*, in variable rates depending on the animal matrix. A significant decrease in erythromycin resistance rate was observed in *E. faecalis* isolated from breeders (-22.7%) in 2023 compared to 2021. A significant decrease of tetracycline resistance (-9.2%) was also observed in *E. faecalis* isolated from broilers in 2023, in comparison to 2021.
Some resistance to antimicrobials critically important for human health was also observed in 2023. Resistance to linezolid was observed in 16 strains, namely 12 *E. faecalis* (11 isolated from veal calves and 1 isolated from pigs) and 4 *E. faecium* (1 isolated from veal calves and 3 isolated from pigs). Additionally, 3 *E. faecalis* isolated breeders (n=1), layers (n=1) and pigs (n=1) were resistant to daptomycine. No resistance to tigecycline, teicoplanin or vancomycin was observed in 2023.

In general, a greater number of multidrug-resistant strains was observed in veal calves (63.3% of *E. faecalis*) and in broilers (53.6% of *E. faecium*). This is similar to previous years. The strains accumulating the most different antimicrobial resistances (n=5 resistances) were *E. faecium* isolated from broilers, breeders and pigs, and *E. faecalis* isolated from veal calves and pigs. A maximum of 7 different resistances was observed in one *E. faecium* isolated from pigs, only. Conversely, a certain percentage of the strains showed no resistance, mainly those isolated from breeding hens, laying hens and pigs (45.3%, 44.8% and 37.5% of *E. faecalis* and 12.5, 43.6% and 16.8% of *E. faecium*, respectively), similarly to what was observed in 2022.

In 2023, an WGS investigation of 19 enterococci was carried out based on the observed phenotypic antimicrobial resistance, i.e., linezolid and daptomycin resistance. The sequencing of these strains provided genetic information regarding their antimicrobial resistance, their typing and their virulence.
Strain typing revealed the identification of 13 different STs, suggesting the presence of some genetic diversity within enterococci isolated from animals exhibiting these phenotypic resistances. However, cgMLST analyses of some strains sharing the same STs revealed likely genetic relatedness between two strains isolated from veal calves and carrying linezolid resistance genes, among others.
Sequencing also revealed the presence of resistance genes explaining the resistant phenotype in 92.6% (n=87/94) of the individual phenotypes observed and allowed to genetically characterize resistances of human interest, such as resistance to critical antibiotics. Linezolid resistance in 2023 was characterized by the presence of transferable genes, as already observed in 2019 (Timmermans *et al.*, 2022) and since 2021 (see FASFC reports). All the resistant strains analyzed (n=16) carried at least one gene, namely *optrA* gene (n=15 with 12 isolated from veal calves and 3 isolated from pigs) or the combination of *optrA*/*poxtA* (n=1 isolated from pigs). In addition to linezolid resistance, these genes also confer resistance to phenicols and phenicols and tetracycline, respectively. Thus, they could be cross-selected through the use of one of the antibiotics to which they confer resistance, other than linezolid (not used in animals). The intrinsic resistance to quinupristin/dalfopristin observed in *E. faecalis* is conferred by the presence of *lsaA*, found in *E. faecalis* isolates only (Torres *et al.,* 2018, Frye & Jackson, 2013). However, mutations present in this gene have been described to induce a loss of function (Singh & Murray, 2005), what could explain the occasional observation of MICs below 1 mg/L, as observed in 4

E. faecalis isolated from broilers (n=1) and layers (n=3) in 2023. The genetic background of the resistance to daptomycin remains unknown. Further research studies should be conducted to decipher this.

Virulence, a common trait of *Enterococcus* spp. was characterized by WGS. Different virulence factors have been described within enterococci, ensuring different functions (adhesion, conjugation, biofilm formation, cytolytic activity, colonization) and allowing them to adapt to their environment. In 2023, the analysis of 19 enterococci revealed the presence of a greater number of virulence factors in *E. faecalis* compared to *E. faecium*, regardless of animal origin. All investigated strains carried at least one virulence factors, *efaAfs/efaAfm* specific for *E. faecalis* and *E. faecium* respectively. Except *agg,* no other virulence factor associated with high pathogenicity and described in human strains (e.g., *esp, cyl*) was found.

4. Conclusion

The antimicrobial resistance monitoring in *Campylobacter coli* and *Campylobacter jejuni* isolated from food samples as well as from samples of broilers, pigs and bovine animals caecal content was carried out in 2023. As it was already detected in previous years, isolates of *Campylobacter coli* are usually more resistant than isolates of *Campylobacter jejuni*. This is especially more evident in the case of resistance to the carbapenem antibiotic class monitored in *Campylobacter* spp., ertapenem. This antibiotic was added in 2021 to the panel of antimicrobials tested and concerning levels of resistance were already detected in *C. coli* in 2021. The results of antimicrobial susceptibility testing in 2023 confirmed these rates of resistances in *C. coli* which were as high as 75% in isolates tested from broilers caecal samples. However, the epidemiological cut-off values recommended by EFSA for the analysis of ertapenem resistance are still under discussion.
An extremely high level of resistance to ciprofloxacin was detected in *C. coli* and a very high level of resistance to this antibiotic was also detected in *C. jejuni* isolated from poultry meat in 2023 and is increasing since 2021. However multi-drug resistance of *Campylobacter* isolates was lower in 2023 than in 2022, 2020 and 2019 in this matrix. In *C. coli* isolated from caecal samples of bovines, resistances to ciprofloxacin, tetracycline and erythromycin were extremely high (94.3%, 96.6% and 76.1% respectively).
Combined resistance to both ciprofloxacin and erythromycin, which are considered critically important for treatment of campylobacteriosis, was not detected in *C. jejuni* isolated from poultry and was low in isolates from bovines.

Salmonella spp. isolated from pigs and bovine animals caecal content were tested for antimicrobial susceptibility in 2023. Since resistance in *Salmonella* spp. is highly dependent on the prevalence of different serotypes, the most prevalent ones were identified for each matrix. In pig caecal content *S.* Typhimurium and its monophasic variant were the most prevalent and showed resistances mostly to ampicillin, tetracycline, sulfamethoxazole, and trimethoprim. Overall, no resistance to 3rd generation cephalosporins, to meropenem, to colistin or to azithromycin was detected in isolates from either pig or bovines caecal content. Only one isolate of *S.* Derby isolated from pig caecal content was resistant to (fluoro)quinolones. In addition, *Salmonella* isolates have been analysed by WGS as well and predicted antimicrobial resistance correlates with phenotypic testing. The gene *aac(6')-Iaa* was found in all genomes independently of the serovars. This gene encodes for a chromosomal aminoglycoside acetyltransferase which confers resistance to aminoglycosides. However, *aac(6')-Iaa* and similar genes usually are transcriptionally silent and rarely become transcriptionally active. The mere presence of this gene does not confer aminoglycoside resistance in *Salmonella* (Neuert *et al.* 2018, Magnet *et al.* 1999).
In 2023, *Salmonella* spp. recovered from food and feed matrices have been analysed by WGS.
Two isolates had a predicted resistance to 3rd generation cephalosporins, one isolated from sunflower seeds belonging to the serotype Kentucky (ST198). This isolate harbored a *bla*$_{\text{CTX-M-14b}}$ together with the following genes: *aac(3)-Id*; *aac(6')-Iaa*, *aadA7*; *aph(3'')-Ib*, *aph(3')-Ia*; *aph(6)-Id*; *sul1*; *tet(A)* which confer a multidrug resistant profile including sulfonamides, tetracycline and gentamicin among other aminoglycosides.
The second isolate harboring a gene conferring resistance to extended spectrum β-lactams was isolated from cut poultry meat and belonged to the serotype Infantis (ST8662). The isolate harbored the following genes: *bla*$_{\text{CTX-M-3}}$, *aac(6')-Iaa; aadA1; aadA1; dfrA14; sul1; tet(A).*
One *Salmonella* Paratyphi B var. Java (ST28) isolated from broiler's neck skin harbored a plasmid mediated colistin resistance gene *mcr-9.1.* The isolate carried the following genes as well*, aac(6')-Iaa; dfrA1; formA; lnu(F); aph(3')-Ia; sul1; qacE* conferring a multidrug resistant profile including trimethoprim, fosfomycin, lincosamide, aminoglycosides, sulfonamides and quaternary ammonium biocides.

The monitoring of antimicrobial resistance in commensal indicator *E.coli* isolated from caecal samples of broilers, fattening pigs and bovine animals under one year taken at the slaughterhouse as well as from fecal samples of beef cattle, laying hens and breeding hens taken at farm level was carried out in 2023. Overall, resistance levels were lower in *E. coli* isolated from food producing animals caecal content in 2023 than in 2022. This is also the case in *E. coli* isolated from fecal samples of beef cattle, laying and breeding hens, sampled at the farm.

Results of antimicrobial susceptibility testing show that the highest prevalences of resistances to 3rd generation cephalosporins, (fluoro)quinolones, ampicillin, gentamicin, sulfamethoxazole and trimethoprim are found in *E. coli* isolated from broilers caecal samples although most of these resistances were lower than those detected in previous years.

Pig caecal content was the only matrix where amikacin resistance was detected in *E. coli* isolates every year since its addition to the test panel in 2021, although a very small number of isolates showed a resistance to it.

Regarding commensal *E. coli* collected at farm level for the three categories mentioned above, we see that the highest resistance to (fluoro)quinolones, ampicillin and tetracycline was detected in breeding hens but resistances to these antibiotics, with the exception of nalidixic acid, were lower in 2023 than in 2022.

Meropenem was the only antimicrobial to which no resistance was detected in 2023 in any food-producing animal category.

In 2023, the specific monitoring of ESBL-AmpC or CP producing *E. coli* from caeca and fresh meat samples from the three animal categories, broilers, fattening pigs and bovines, have revealed a significant decrease in the prevalence of ESBL *E. coli* isolated from broilers at slaughterhouse and fresh meat derived therefrom at retail. In line with previous years, the prevalence of ESBL *E. coli* on broiler fresh meat was the highest (45.51%) among the fresh meat categories followed by bovine fresh meat (3.19%) and pig meat (1.92%), which remains low. Concerning food producing animal categories, ESBL *E. coli* was more prevalent in bovine animals under one year (66.67%) than in broilers (61.72%) for the first time, and followed by fattening pigs (29.26%). No meropenem-resistant isolates were detected in none of the matrices tested, in line with previous years. Genomic characterization of ESBL-AmpC producing *E. coli* isolates has reported the following genes encoding for ESBL enzymes *bla*$_{\text{SHV-12}}$, *bla* $_{\text{CTX-M-55 and}}$ *bla* $_{\text{TEM-52}}$ as the most frequent found in isolates from broilers sampled at slaughterhouse and fresh poultry meat sampled at retail. Isolates from bovines and fattening pigs carried genes encoding for enzyme production belonging to the CTX-M group 1. In addition, a significant number of isolates from bovines carried the gene *bla*$_{\text{CTX-M-2}}$, suggesting this category of food producing animals as a reservoir of this particular mechanism of resistance.

Predicted resistance to (fluoro)quinolones, considering all the resistance determinants including chromosomal mutations, was observed in 57.49%, 38.12% and 22.22% of ESBL *E. coli* isolates from broilers, bovines and fattening pigs, respectively.

Resistance to colistin among the ESBL-AmpC producing *E. coli* isolates remains rare. Predicted sensitivity to this antimicrobial was detected in 100%, 98,88% and in 98,99% of *E. coli* ESBL isolates from broilers, fattening pigs and bovines. Three acquired resistance genes associated to phenotypic resistance to colistin were found. The *mcr 2.1* was found in one isolate from caecal content of fattening pigs and the other isolates harboring a *mcr 1.1* gene were recovered from caecal content from bovines, however only *bla*$_{\text{TEM-135}}$ was found in those isolates suggesting that other mechanisms of resistance not know may confer resistance to extended spectrum β-lactams. All isolates from fresh meat, were predicted to be susceptible to colistin except one recovered from fresh turkey meat which harbored the associated gene *mcr 1.1*, together with *bla*$_{\text{CMY-2,}}$ *tet(A), bla*$_{\textit{TEM-1B}}$*, floR,* and point mutation in the genes *gyrA p.S83L, gyrA p.D87N, parC p.S80I* and *parE p.S458A* conferring a multidrug resistant profile including besides colistin, 3rd generation cephalosporins, tetracycline, phenicols and (fluoro)quinolones.

The presence of MRSA in food-producing animals and their carriage of several AMR and virulence genes represents a public health risk. The prevalence was very low in broilers, moderate in fattening turkeys and null in laying hens in 2023, which is stable compared to previous years. All 6 isolates (n=1 from broilers, n=5 from fattening turkey isolates) were analysed by WGS and genotyped as LA-MRSA according to their ST/*spa*-types combinations.

All MRSA isolated in 2023 were harboring the *mecA* gene and the *tet(M)* tetracycline resistance gene , which are also characteristics of LA-MRSA. Several other resistance genes were observed and detailed in the results. All the 6 MRSA isolates were genetically multi-drug resistant (i.e., carrying genes conferring resistance to at least 3 different antibiotic classes). However, in 2023, no gene encoding resistance to the critically important antibiotics (linezolid and vancomycin) was detected.

Moreover, several virulence genes associated with the immune evasion cluster (*sak, scn*), associated with toxins (*hlgA, hlgB, hlgC* and *selw*) and/or exoenzymes (*aur*) were detected among the 6 MRSA isolates analysed in 2023. One LA-MRSA isolate from fattening turkeys carried the *sak* and *scn* genes associated with the human immune evasion cluster and several genes associated with toxins (*hlgA*, *hlgB*, *hlgC*, and *selw*) and exoenzyme (*aur*). This isolate would probably not have been recently transmitted from humans to turkeys, given the livestock-associated genetic background and the carriage of the *tet(M)* gene. The genes associated with the immune evasion cluster were not observed in the other isolates.

In 2023, the monitoring of *Enterococcus faecalis* and *Enterococcus faecium* isolated from food-producing animals continued. The prevalences of enterococci species by animal category observed this year were similar to those observed in previous years. Indeed, *Enterococcus faecium* was more frequently isolated than *Enterococcus faecalis* within the samples of breeding hens (88.4%), laying hens (72.0%), veal calves (54.8%) and pigs (62.3%). Conversely, *E. faecalis* was isolated more frequently than *E. faecium* in broiler (68.8%) samples. The antimicrobial susceptibility tests carried out this year showed that, in general, the resistance rates observed in *Enterococcus faecalis* and *Enterococcus faecium* within the different animal matrices studied have remained stable since 2019, with the exception of significant decreases in the resistance rate to certain antimicrobials. A significant decrease in erythromycin resistance rate was observed in *E. faecalis* isolated from breeders (-22.7%) in 2023 compared to 2021. A significant decrease of tetracycline resistance (-9.2%) was also observed in *E. faecalis* isolated from broilers in 2023, in comparison to 2021. Resistance to tetracycline, erythromycin and to quinupristin/dalfopristin were still the most observed resistances, both in *E. faecalis* and *E. faecium*, in variable rates depending on the animal matrix Some resistance to antimicrobials critically important for human health was also observed in 2023. Resistance to linezolid was observed in 16 strains, namely 12 *E. faecalis* (11 isolated from veal calves and 1 isolated from pig) and 4 *E. faecium* (1 isolated from veal calve and 3 isolated from pigs). Additionally, 3 *E. faecalis* isolated breeders, (n=1) layers (n=1) and pigs (n=1) were resistant to daptomycine. No resistance to teicoplanin, tigecycline or vancomycin was observed in 2023.

In general, a greater number of multidrug-resistant strains was observed in veal calves (63.3% of *E. faecalis)* and in broilers (53.6% of *E. faecium*). This is similar to previous years. The strains accumulating the most different antimicrobial resistances were those isolated from broilers, breeders, pigs, and from veal calves, with a maximum of 7 different resistances observed in one *E. faecium* isolated from pigs.

A WGS investigation of 19 enterococci resistant to linezolid and/or daptomycin was carried out in order to genetically characterize the antimicrobial resistance observed. This revealed the genetic diversity of the strains studied with 13 different STs identified and a variable number of virulence factors (from 1 to 17 different factors) depending on the bacterial species. Besides, close relatedness between two linezolid resistant strains isolated from veal calves was also observed. The *agg* virulence factor gene associated with high pathogenicity in human strains was detected in some animal strains, which is a

matter of concern. Complementary to phenotyping, sequencing enable to identify the resistance mechanisms within enterococci isolated from animals. Indeed, the presence of resistance genes explaining the resistant phenotype was observed in 95.6% (n=87/91) of the individual phenotypes observed. Resistance to linezolid was characterized by the presence of the *optrA* and *poxtA* genes, two genes that could be cross-selected through the use of either phenicols or tetracycline, given that linezolid is not used in animals. The genetic background of the resistance to daptomycin remains unknown. Further research studies should be conducted to decipher this.

Taking together all results from both gram-negative (*E. coli*) and gram-positive indicators (enterococci) isolated from food-producing animals, resistance rates showed overall a decrease in 2023 in *E. coli* and a global status quo in enterococci (except 2 particular significant decreases).

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Annex Ia Ib Ic WGS MRSA 2023

8. Abbreviations

AmpC : AmpC-type cephalosporins
AMR : Antimicrobial Resistance
CA-MRSA : community-associated MRSA
cgMLST : core-genome MLST
ESBL : Extended Spectrum B-Lactamase
EURL-AR : European Reference Laboratory for Antimicrobial Resistance
HA-MRSA: hospital-associated MRSA
LA-MRSA: livestock-associated MRSA
MDR : multi-drug resistance
MIC : Minimal Inhibitory Concentration
MLST : Multi Locus Sequence Typing
ST : sequence-type

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