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 2022

2022 Report

National Referance Laboratory for antimicrobial resistance

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* and *Campylobacter* and in methicillin-resistant *Staphylococcus aureus* (MRSA) as well as resistance in indicator bacteria *Escherichia coli*, *Enterococcus faecalis* and *Enterococcus faecium* was monitored in 2022. Moreover, a specific monitoring of presumptive extended spectrum beta-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 2022, 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 (EUVSEC3/EUCAMP3).

In *Campylobacter jejuni* isolated from poultry meat, the predominant resistance profiles included ciprofloxacin combined with tetracycline. An increase in resistance to these antibiotics as well as to ertapenem and erythromycin was seen in 2022. Multidrug resistance in *C. jejuni* also increased in 2022 up to 13% compared to 4,4% in 2021. In *Campylobacter coli* from poultry meat, while ciprofloxacin resistance increased in 2022, resistance to erythromycin, ertapenem and tetracycline slightly decreased, thus lowering the multidrug resistance rate as well from 43,48% in 2021 to 39,13%.
In primary production, the monitoring of *Campylobacter coli* in fattening pigs showed overall stable resistance levels except for an increased resistance to erythromycin and a first detection of low chloramphenicol resistance. In 2022, 20% of the isolates were resistant to the macrolide erythromycin compared to 9% observed in 2021. However, in *C. coli* isolated from veal calves, resistances to erythromycin, ertapenem and chloramphenicol were lower in 2022 than in 2021 but resistances to ciprofloxacin and gentamicin were higher. In *C. jejuni*, resistances to erythromycin, ertapenem, gentamicin and chloramphenicol increased but resistances to ciprofloxacin and tetracycline remained stable. Overall, as seen in previous reports, antimicrobial resistance levels are lower in *C. jejuni* than in *C. coli*.

In 2022, considering all *Salmonella* spp. recovered from food matrices, the most prevalent serotypes were *S.* Infantis recovered mainly from fresh meat from poultry and *S.* Monophasic Typhimurium from fattening pigs and veal calves. Resistance levels increased for 6 out of the 15 tested antimicrobials in *Salmonella* isolated from food matrices, resistance to amikacin was also detected for the first time in processed meat (cold cuts) but resistance to (fluoro)quinolones decreased compared to 2021. In food-producing animals however, resistance to (fluoro)quinolones increased in 2022, especially in poultry. Resistance to third generation cephalosporins was only found in one isolate of *S*. Paratyphi B var L(+) tartrate(+) from poultry cutted meat and one *S*. Dublin recovered from veal calves. Overall, resistance to colistin is very low, only two isolates of *S*. Dublin recovered one from poultry cutted meat and another from veal calves were resistant to colistin.

The specific monitoring of ESBL, AmpC or carbapenemase producing *E.coli* was carried out in broilers, turkeys, fattening pigs and veal calves and in meat derived from these 4 categories of animals. As it has been the case in the previous years, the highest prevalence of ESBL *E.coli* was found in broilers (75%) followed by turkeys (64%), veal calves (64%) and fattening pigs (35%). In fresh meat from food-producing animals as well, the prevalence was the highest in fresh meat from broilers (58%) followed by fresh meat from turkeys (24%). The prevalence was much lower in fresh meat from veal calves (2%) and fresh meat from fattening pigs (2%). No meropenem-resistant isolates were detected in 2022. However, ESBL *E.coli* isolated from these matrices showed extremely high levels of multidrug resistance (>80%).

In indicator *E.coli*, in comparison with 2021 an overall increased resistance was detected in most categories of food-producing animals, especially in isolates from faeces samples of laying hens, breeding hens and bovines at the farm level. (Fluoro)quinolones resistance also increased in isolates from caecal samples of broilers, veal calves, and fattening pigs taken at the slaughterhouse. However, resistance to critical antimicrobials such as colistin, tigecycline, cephalosporins and meropenem remains very low or non-existant in the different matrices in 2022. Also, in *E.coli* isolated from caecal samples of fattening turkeys, the levels of resistance to most antimicrobials were lower in 2022 than in 2021.

Monitoring of methicillin-resistant *Staphylococcus aureus* (MRSA) was carried out in fattening pigs and sows on farm in 2022. The bovines and poultry were monitored in 2021 and 2020, 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 170 of collected MRSA isolates together with their AMR and virulence genes. Several changes in the methodology used for the monitoring of MRSA have been made in 2022, including a new isolation method and the study of AMR through NGS rather than phenotypic susceptibility testings. The 2022 data will now serve as a new baseline for analyzing future trends. The extremely high prevalence (87.9%) observed in fattening pigs and, in a lesser extent, the very high prevalence observed in sows (52.6%), in 2022 with the new isolation method is a matter of concern.
In 2022, all but one isolate were genotyped as LA-MRSA according to their STs/spa-types combinations. The latter isolate is likely to belong to LA-MRSA as well, according to our investigation. All MRSA isolated were harboring the *mecA* gene and at least one tetracycline resistance genes, which are also characteristic of LA-MRSA. Several other resistance genes were observed. Of particular concern is the carriage of the *cfr* gene, encoding a.o. resistance to the critically important antibiotic linezolid, observed again in 2022 in 3 isolates (n=1 sows and n=2 fattening pigs). In addition to AMR genes, *qac* (precisely, *qacG* and *qacJ*) genes mediating resistance to quaternary ammonium compounds were observed in some MRSA isolates in 2022. Moreover, several virulence genes associated with the immune evasion cluster (*sak, scn*), toxins (*hlgA, hlgB, hlgC, seb* and *selw*) and exoenzymes (*aur*) were detected. One sow isolate belonging to the ST398-t034 LA-MRSA type 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 did not carry critically antimicrobial resistance gene (no *cfr* gene), neither *qac* disinfectant resistance gene. The genes associated with the immune evasion cluster were not observed in the other isolates. The detection of the *seb* gene encoding an exotoxin known to be the source for multiple pathologies in humans in an isolate from fattening pigs highlights the importance of monitoring these different virulence factors in the future. 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 2022, 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 (85.6%), laying hens (69.9%), veal calves (64.0%) and pigs (66.4%). Conversely, *E. faecalis* was isolated more frequently than *E. faecium* in broiler (65.4%) and turkey (92.0%) 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 for chloramphenicol in *E. faecalis* isolated from veal calves, and for ampicillin in *E. faecium* isolated from broilers in 2022. 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=18), a critical antibiotic for human health, was also observed in 2022, in 13 *E. faecalis* isolated from veal calves (n=11), pigs (n=2) and in 5 *E. faecium* isolated from pigs (n=2), veal calves (n=2) and breeding hens (n=1). No resistance to teicoplanin or vancomycin was observed in 2022. Daptomycin resistance was very low in 2022 but it could be related to the modification of its threshold in 2021. Tigecycline resistance was observed for the first time since 2019 (n=2). Multidrug resistance (resistance to at least 3 antimicrobial classes) was mainly observed in broilers and veal calves with 60.3% of multi-resistant *E. faecium* and 60.2% of multi-resistant *E. faecalis*, respectively. In addition, strains accumulating the higher number of different antimicrobial resistances were isolated from veal calves, with a maximum of 6 different resistances observed in *E. faecalis* and *E. faecium*.

In 2022, some enterococci were completely sequenced by Next-generation sequencing (NGS). This report also presents these NGS results and the detailed characterization of these strains, including the detection of *optrA* and *poxtA* genes encoding linezolid resistance.

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* et *Campylobacter* et celle du *Staphylococcus aureus* résistant à la méticilline (MRSA) ont fait l'objet d'un monitoring en 2022, de même que la résistance des bactéries indicatrices *Escherichia coli*, *Enterococcus faecalis* et *Enterococcus faecium*. En outre, un monitoring spécifique des *E.coli* productrices présumées de bêta-lactamases à spectre étendu /AmpC/carbapénémase 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 2022, 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 d'antimicrobiens (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. Une augmentation de la résistance à ces antibiotiques ainsi qu'à l'ertapénème et à l'érythromycine a été observée en 2022. La multirésistance de *C. jejuni* a également augmenté en 2022, atteignant ainsi 13% contre 4,4% en 2021. Chez les *Campylobacter coli* isolés à partir de viandes de volailles, tandis que la résistance à la ciprofloxacine a augmenté en 2022, la résistance à l'érythromycine, à l'ertapénème et à la tétracycline a légèrement diminué, faisant ainsi baisser le taux de multirésistance à 39,13%, contre 43,48% en 2021. Dans la production primaire, le monitoring de *Campylobacter coli* chez les porcs d'engraissement a montré des niveaux de résistance globalement stables, à l'exception d'une résistance accrue à l'érythromycine et d'une première détection de faible résistance au chloramphénicol. En 2022, 20% des isolats étaient résistants à l'érythromycine, un macrolide, contre 9% en 2021. Toutefois, chez les *C. coli* isolés à partir de veaux de boucherie, les résistances à l'érythromycine, à l'ertapénème et au chloramphénicol étaient plus faibles en 2022 qu'en 2021, mais les résistances à la ciprofloxacine et à la gentamicine étaient plus élevées. Chez *C. jejuni*, les résistances à l'érythromycine, à l'ertapénème, à la gentamicine et au chloramphénicol ont augmenté, mais les résistances à la ciprofloxacine et à la tétracycline sont restées stables. 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 2022, si l'on considère tous les isolats de *Salmonella* spp. provenant de matrices alimentaires, les sérotypes les plus répandus étaient le sérotype *S.* Infantis provenant principalement de viandes fraîches de volailles et le sérotype *S.* Typhimurium monophasique provenant de porcs d'engraissement et de veaux de boucherie. Les niveaux de résistance ont augmenté pour 6 des 15 antimicrobiens testés dans les isolats de *Salmonella* provenant de matrices alimentaires. La résistance à l'amikacine a également été détectée pour la première fois dans de la charcuterie, tandis que la résistance aux (fluoro)quinolones a diminué par rapport à 2021. Cependant, la résistance aux (fluoro)quinolones a augmenté en 2022 chez les animaux producteurs de denrées alimentaires, en particulier chez les volailles. La résistance aux céphalosporines de troisième génération n'a été détectée que dans un isolat de *S*. Paratyphi B var. L(+) tartrate(+) provenant de viandes découpées de volailles et un isolat de *S*. Dublin provenant de veaux de boucherie. Dans l'ensemble, la résistance à la colistine est très faible. Seuls deux isolats de *S*. Dublin – provenant d'une part de viandes de volailles et d'autre part de veaux de boucherie – étaient résistants à la colistine.

Le monitoring spécifique des *E.coli* productrices de BLSE, d'AmpC ou de carbapénémase a été réalisé chez les poulets de chair, les dindes, les porcs d'engraissement et les veaux de boucherie, ainsi que dans les viandes dérivées de ces 4 catégories d'animaux. Comme les années précédentes, la plus forte prévalence d'*E.coli* BLSE a été observée chez les poulets de chair (75%), suivis des dindes (64%), des

veaux de boucherie (64%) et des porcs d'engraissement (35%). Concernant les viandes fraîches provenant d'animaux producteurs de denrées alimentaires aussi, la prévalence était la plus forte dans les viandes fraîches de poulets de chair (58%), suivies des viandes fraîches de dindes (24%). La prévalence était beaucoup plus faible dans les viandes fraîches de veaux de boucherie (2%) et de porcs d'engraissement (2%). Aucun isolat résistant au méropénème n'a été détecté en 2022. Cependant, les *E.coli* BLSE isolées dans ces matrices ont montré des niveaux de multirésistance extrêmement élevés (>80%).

Concernant les *E.coli* indicatrices, en comparaison avec 2021, une résistance globale accrue a été détectée dans la plupart des catégories d'animaux producteurs de denrées alimentaires, en particulier dans les isolats provenant d'échantillons de fèces de poules pondeuses, de poules reproductrices et de bovins au niveau de l'exploitation. La résistance aux (fluoro)quinolones a également augmenté dans les isolats provenant d'échantillons cæcaux de poulets de chair, de veaux de boucherie et de porcs d'engraissement prélevés à l'abattoir. Toutefois, la résistance aux antimicrobiens critiques tels que la colistine, la tigécycline, les céphalosporines et le méropénème demeure très faible, voire inexistante, dans les différentes matrices en 2022. Par ailleurs, chez les *E.coli* isolées à partir d'échantillons cæcaux de dindes d'engraissement, les niveaux de résistance à la plupart des antimicrobiens étaient plus faibles en 2022 qu'en 2021.

En 2022, un monitoring du *Staphylococcus aureus* résistant à la méticilline (MRSA) a été effectué chez les porcs d'engraissement et les truies au sein des exploitations. Les bovins et volailles ont fait l'objet d'un monitoring en 2021 et 2020, respectivement (rotation de 3 ans). L'objectif de ce monitoring est d'évaluer la prévalence du MRSA dans ces catégories d'animaux et de déterminer les génotypes (ST et *spa* type) de 170 isolats de MRSA collectés, ainsi que leurs gènes de RAM et de virulence. En 2022, plusieurs changements ont été apportés à la méthodologie utilisée pour le monitoring du MRSA, notamment une nouvelle méthode d'isolement et une étude de la RAM par le biais du NGS plutôt que par le biais de tests de susceptibilité phénotypique. Les données de 2022 serviront désormais de nouvelle base de référence pour l'analyse des tendances futures. La prévalence extrêmement élevée (87,9%) observée chez les porcs d'engraissement et, dans une moindre mesure, la prévalence très élevée observée chez les truies (52,6%) en 2022 via la nouvelle méthode d'isolement constituent un sujet de préoccupation.
En 2022, tous les isolats sauf un ont été génotypés comme LA-MRSA (MRSA associé à l'élevage) selon leurs combinaisons ST/*spa* types Selon notre investigation, ce dernier isolat est susceptible d'appartenir lui aussi au génotype LA-MRSA. Tous les isolats de MRSA étaient porteurs du gène *mecA* et d'au moins un gène de résistance à la tétracycline, ceux-ci étant également caractéristiques des LA-MRSA. Plusieurs autres gènes de résistance ont été observés. Le portage du gène *cfr*, codant entre autres pour la résistance à l'antibiotique critique linézolide et encore observé en 2022 dans 3 isolats (n=1 truies et n=2 porcs d'engraissement), est particulièrement préoccupant. Outre les gènes de RAM, les gènes *qac* (plus précisément, *qacG* et *qacJ*) médiateurs de la résistance aux composés d'ammonium quaternaire ont été observés dans plusieurs isolats de MRSA en 2022. De plus, plusieurs gènes de virulence associés au cluster d'évasion immunitaire (*sak, scn*), à des toxines (*hlgA, hlgB, hlgC, seb* et *selw*) et à des exoenzymes (*aur*) ont été détectés. Un isolat de truies appartenant au type LA-MRSA ST398-t034 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 ne portait pas de gène de résistance aux antimicrobiens critiques (pas de gène *cfr*), ni de gène de résistance aux désinfectants (*qac*). Les gènes associés au cluster d'évasion immunitaire n'ont pas été observés dans les autres isolats. Le fait que le gène *seb* codant pour une exotoxine connue pour être la source de multiples pathologies humaines ait été détecté dans un isolat provenant de porcs d'engraissement souligne l'importance du monitoring de ces différents facteurs de virulence dans le futur. 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, qui a ensuite repris en 2019, a été poursuivi.

L'étude de la prévalence de la RAM dans 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 2022, 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 (85,6%), de poules pondeuses (69,9%), de veaux de boucherie (64,0%) et de porcs (66,4%). Inversement, l'espèce *E. faecalis* a été isolée plus souvent que l'espèce *E. faecium* dans les échantillons de poulets de chair (65,4%) et de dindes (92.0%).
Les tests de susceptibilité aux antimicrobiens 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). Des baisses significatives de la résistance aux antimicrobiens ont été observées pour le chloramphénicol chez *E. faecalis* isolées d'échantillons de veaux de boucherie et pour l'ampicilline chez *E. faecium* isolées de poulets de chair en 2022. 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=18), un antibiotique critique pour la santé humaine, a également été observée en 2022 dans 13 isolats d'*E. faecalis* provenant de veaux de boucherie (n=11) et de porcs (n=2) et dans 5 isolats d'*E. faecium* provenant de porcs (n=2), veaux de boucherie (n=2) et de poules reproductrices (n=1). Aucune résistance à la teicoplanine ou à la vancomycine n'a été observée en 2022. La résistance à la daptomycine était très faible en 2022, mais cela pourrait être dû à la modification de son seuil en 2021. La résistance à la tigécycline a été observée pour la première fois depuis 2019 (n=2). Une multirésistance (résistance à au moins 3 classes d'antimicrobiens) a principalement été observée chez les poulets de chair et les veaux de boucherie avec respectivement 60,3% d'*E. faecium* multirésistantes et 60,2% d'*E. faecalis* multirésistantes. En outre, les souches accumulant le plus grand nombre de résistances antimicrobiennes différentes ont été isolées chez les veaux de boucherie, avec un maximum de 6 résistances différentes observées chez *E. faecalis* et *E. faecium*.

En 2022, certaines entérocoques ont été complètement séquencées par séquençage de nouvelle génération (NGS). Ce rapport présente également les résultats du NGS et la caractérisation détaillée de ces souches, y compris la détection des gènes *optrA* et *poxtA* codant pour la résistance au linézolide.

Abstract

In België volgt het FAVV de evolutie van antimicrobiële resistentie (AMR) op in voeding en voedselproducerende dieren (primaire productie). In 2022 werd de resistentie in de zoönotische bacteriën *Salmonella* en *Campylobacter* en in meticillineresistente *Staphylococcus aureus* (MRSA), evenals resistentie in de indicatorbacteriën *Escherichia coli*, *Enterococcus faecalis* en *Enterococcus faecium* gecontroleerd. Bovendien wordt een specifieke monitoring van vermoedelijke Extended spectrum bèta-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-off waarden (ECOFF) volgens EUCAST (European Committee on Antimicrobial Susceptibility Testing).

In 2022 werd het Uitvoeringsbesluit 2020/1729 van de Europese Commissie van 17 november 2020 van toepassing voor het bepalen van de epidemiologische cut-off waarden en voor de selectie van antimicrobiële panels (EUVSEC3/EUCAMP3).

Campylobacter jejuni, geïsoleerd uit vlees van gevogelte, bevatte de overheersende resistentieprofielen ciprofloxacine in combinatie met tetracycline. Een toename in de resistentie tegen deze antibiotica en tegen ertapenem en erytromycine werd in 2022 aangetroffen. Multiresistentie tegen geneesmiddelen in *C. jejuni* nam ook toe in 2022 tot 13% vergeleken met 4,4% in 2021. Bij *Campylobacter coli* uit pluimveevlees nam de resistentie tegen ciprofloxacine toe in 2022, maar nam de resistentie tegen erytromycine, ertapenem en tetracycline licht af, waardoor ook de multiresistentie tegen geneesmiddelen daalde van 43,48% in 2021 naar 39,13%.
In de primaire productie toonde de monitoring van *Campylobacter coli* in vleesvarkens over het algemeen stabiele resistentieniveaus, behalve een verhoogde resistentie tegen erytromycine en een eerste detectie van een lage resistentie tegen chlooramfenicol. In 2022 was 20% van de isolaten resistent tegen het macrolide erytromycine, vergeleken met 9% in 2021. In *C. coli* geïsoleerd uit vleeskalveren waren de resistenties tegen erytromycine, ertapenem en chlooramfenicol echter lager in 2022 dan in 2021, maar de resistenties tegen ciprofloxacine en gentamicine waren hoger. Bij *C. jejuni* nam de resistentie tegen erytromycine, ertapenem, gentamicine en chlooramfenicol toe, maar bleef de resistentie tegen ciprofloxacine en tetracycline stabiel. Over het algemeen zijn, zoals in eerdere rapporten vermeld, de antimicrobiële resistentieniveaus lager bij *C. jejuni* dan bij *C. coli*.

In 2022, rekening houdend met alle *Salmonella* spp. uit voedselmatrices, waren *S.* Infantis, voornamelijk uit vers vlees van gevogelte, en *S.* Monophasic Typhimurium uit vleesvarkens en vleeskalveren de meest gangbare serotypes. Het resistentieniveau steeg voor 6 van de 15 geteste antimicrobiële stoffen in *Salmonella* geïsoleerd uit voedselmatrices, resistentie tegen amikacine werd ook voor het eerst gedetecteerd in vleeswaren, maar de resistentie tegen (fluoro)chinolonen daalde ten opzichte van 2021. Bij voedselproducerende dieren nam echter de resistentie tegen (fluor)chinolonen toe in 2022, vooral bij pluimvee. De resistentie tegen cefalosporines van de derde generatie werd enkel in één isolaat van *S*. Paratyphi B var L(+) tartraat(+) uit versneden vlees van gevogelte en in één isolaat van *S*. Dublin uit vleeskalveren aangetroffen. Over het algemeen is de resistentie tegen colistine zeer laag, slechts twee isolaten van *S*. Dublin, één uit versneden vlees van gevogelte en één uit vlees van vleeskalveren waren resistent tegen colistine.

De specifieke monitoring van ESBL-, AmpC- of carbapenemaseproducerende *E.coli* werd uitgevoerd bij braadkippen, kalkoenen, vleesvarkens en vleeskalveren en in vlees afkomstig van deze 4 categorieën dieren. Net als in de voorgaande jaren werd de hoogste prevalentie van ESBL *E.coli* aangetroffen bij braadkippen (75%), gevolgd door kalkoenen (64%), vleeskalveren (64%) en vleesvarkens (35%). Ook in vers vlees van voedselproducerende dieren was de prevalentie het hoogst in vers vlees van braadkippen (58%), gevolgd door vers vlees van kalkoenen (24%). De prevalentie was veel lager in vers vlees van vleeskalveren (2%) en in vers vlees van vleesvarkens (2%). In 2022 werden geen

isolaten gedetecteerd die resistent waren tegen meropenem. Echter, ESBL-*E.coli* geïsoleerd uit deze matrices vertoonde extreem hoge niveaus van multidrugresistentie (>80%).

Bij indicator-*E.coli* werd, in vergelijking met 2021, een algemeen verhoogde resistentie vastgesteld bij de meeste categorieën voedselproducerende dieren, vooral bij isolaten uit fecesmonsters van legkippen, fokhennen en runderen op bedrijfsniveau. De resistentie tegen (fluor)chinolonen nam ook toe in isolaten uit caecummonsters van braadkippen, vleeskalveren en vleesvarkens, genomen in het slachthuis. In 2022 blijft echter de resistentie tegen kritische antimicrobiële stoffen zoals colistine, tigecycline, cefalosporines en meropenem zeer laag of afwezig in de verschillende matrices. Ook bij *E.coli* geïsoleerd uit caecummonsters van vleeskalkoenen waren de resistentieniveaus tegen de meeste antimicrobiële stoffen lager in 2022 dan in 2021.

Monitoring van meticillineresistente *Staphylococcus aureus* (MRSA) werd uitgevoerd bij vleesvarkens en zeugen op de hoeve in 2022. De runderen en het pluimvee werden in respectievelijk 2021 en 2020 (3-jaarlijkse rotatie) gecontroleerd. Het doel van deze monitoring is het beoordelen van de MRSA-prevalentie in deze diercategorieën en het bepalen van de genotypen (ST's en *spa*-typen) van 170 van de verzamelde MRSA-isolaten samen met hun AMR- en virulentiegenen. In 2022 zijn er verschillende wijzigingen doorgevoerd in de methodologie die wordt gebruikt voor het monitoren van MRSA, waaronder een nieuwe isolatiemethode en het bestuderen van AMR door middel van NGS in plaats van fenotypische gevoeligheidstests. De gegevens van 2022 zullen nu dienen als een nieuwe basislijn voor het analyseren van toekomstige trends. De extreem hoge prevalentie (87,9%) die werd waargenomen bij vleesvarkens en, in mindere mate, de zeer hoge prevalentie die werd waargenomen bij zeugen (52,6%), in 2022 met de nieuwe isolatiemethode, is reden tot bezorgdheid.
In 2022 werden op één na alle isolaten gegenotypeerd als LA-MRSA (met vee geassocieerde MRSA) volgens hun combinaties ST/*spa*-types. Volgens ons onderzoek behoort het laatste isolaat waarschijnlijk ook tot LA-MRSA. Alle geïsoleerde MRSA's droegen het *mecA*-gen en ten minste één tetracycline-resistentiegen, die ook kenmerkend zijn voor LA-MRSA. Er werden verschillende andere resistentiegenen waargenomen. Bijzonder zorgwekkend is die een *cfr*-gen dragen, dat codeert voor o.a. resistentie tegen het uiterst belangrijke antibioticum linezolid, dat opnieuw waargenomen is in 2022 in 3 isolaten (n=1 zeugen en n=2 vleesvarkens). Naast AMR-genen werden in 2022 in sommige MRSA-isolaten *qac*-genen (om precies te zijn *qacG*- en *qacJ*-) waargenomen die resistentie tegen quaternaire ammoniumverbindingen coderen. Bovendien werden verschillende virulentiegenen in verband gebracht met de immuunevasiecluster *(sak, scn*), toxines *(hlgA, hlgB, hlgC, seb* en *selw*) en exo-enzymen *(aur*) gedetecteerd. Eén isolaat bij zeugen die behoort tot het ST398-t034 LA-MRSA-type bevatte de *sak*- en *scn*-genen in verband met de humane immuunevasiecluster en verschillende genen in verband met de toxines *(hlgA, hlgB, hlgC* en *selw*) en het exo-enzym *(aur*). Dit isolaat bevatte geen kritisch antimicrobieel resistentiegen (geen *cfr*-gen), noch een *qac*-resistentiegen tegen desinfectiemiddelen. In de andere isolaten werden er geen genen in verband met de immuunevasiecluster waargenomen. De detectie van het *seb*-gen dat codeert voor een exotoxine, waarvan bekend is dat het de bron is van meerdere pathologieën bij de mens, in een isolaat van vleesvarkens benadrukt het belang van het monitoren van deze verschillende virulentiefactoren in de toekomst. De aanwezigheid van MRSA in voedselproducerende dieren en hun dragerschap van verschillende AMR- en virulentiegenen vormt een risico voor de volksgezondheid.

Het monitoren 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 2022 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 (85,6%), legkippen (69,9%), vleeskalveren (64,0%) en varkens (66,4%). Daarentegen werd *E. faecalis* vaker geïsoleerd dan *E. faecium* in monsters van braadkippen (65,4%) en kalkoenen (92,0%).

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 waargenomen voor chloramfenicol in *E. faecalis* geïsoleerd uit vleeskalveren, en voor ampicilline in *E. faecium* geïsoleerd uit braadkippen in 2022. 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=18), een cruciaal antibioticum voor de menselijke gezondheid, werd ook in 2022 waargenomen, bij 13 *E. faecalis* geïsoleerd uit vleeskalveren (n=11), varkens (n=2) en bij 5 *E. faecium* geïsoleerd uit varkens (n=2), vleeskalveren (n=2) en fokhennen (n=1). In 2022 werd geen resistentie tegen teicoplanine of vancomycine waargenomen. Daptomycineresistentie was zeer laag in 2022, maar dit kan verband houden met de wijziging in de drempelwaarde ervan in 2021. Tigecyclineresistentie werd voor het eerst sinds 2019 waargenomen (n=2). Multidrugresistentie (resistentie tegen ten minste 3 antimicrobiële klassen) werd voornamelijk waargenomen bij braadkippen en vleeskalveren met respectievelijk 60,3% multiresistente *E. faecium* en 60,2% multiresistente *E. faecalis*. Bovendien werden stammen met het hoogste aantal verschillende antimicrobiële resistenties geïsoleerd uit vleeskalveren, met een maximum van 6 verschillende resistenties die werden waargenomen bij *E. faecalis* en *E. faecium*.

In 2022 werden sommige enterokokken volledig gesequenced met behulp van Next-generation sequencing (NGS). Dit rapport geeft ook deze NGS-resultaten en de gedetailleerde karakterisering van deze stammen weer, met inbegrip van de detectie van *optrA*- en *poxtA*-genen die coderen op linezolidresistentie.

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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 beta-lactamases (ESBL) producing bacteria, i.e. enzymes that confer resistance (or a reduction of activity) to a wide range of beta-lactam antibiotics, including third generation cephalosporins.

Resistant bacteria have differents 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 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, modifying some antibiotics used in the test panels and some epidemiological cutt-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 slaugterhouse are also part of the monitoring. These indicator bacteria can contaminate food. Their resistance levels represent 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 2022, the monitoring focused on fattening pigs and sows. The aim of this monitoring is to assess the MRSA prevalence on farms and determine the genotypes (STs and spa-types) of 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 2022 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 2022. 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*.

The use of NGS was implemented in 2021 as part of official monitoring to investigate antimicrobial resistant bacteria isolated from 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 NGS (Next Generation Sequencing). These machines 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 NGS on a voluntary basis for the detection of ESBL/AmpC/carbapenemase-producing *E. coli* replace panels 1 and 2 of the phenotypic antimicrobial susceptibility testing method in the monitoring of these organisms (EU 2020/1729).
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 2022, 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 *E.coli* and *Salmonella*, EUCAMP3 for *Campylobacter*, and EUVENC for *Enterococcus faecalis* and *Enterococcus faecium*. The interpretaion 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 2022.

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 2022.

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 2022.

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 2022.

Table 5. Panel of antimicrobials tested (first panel EUVSEC3) and interpretation thresholds for indicator and ESBL *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 2022.

Table 6. Panel of antimicrobials tested (second panel EUVSEC2) and interpretation thresholds for indicator and ESBL *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 2022.

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	0.5[#]	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 0.5 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* isolates with resistance to third generation cephalosporins. The criteria were updated in 2016 and presented at the EFSA AMR-Network meeting in November 2016 (see Table 8).

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

Case	Phénotype	Description
1	ESBL	Cefotaxime or ceftazidime > 1mg/L (R) Cefotaxime/clavulanic acid or ceftazidime/clavulanic acid synergy Cefoxitin < 8mg/L (S)
2	AmpC	Cefoxitin > 8mg/L (R) No cefotaxime/clavulanic acid or ceftazidime/clavulanic acid synergy
3	ESBL+AmpC	Cefotaxime or ceftazidime > 1mg/L (R) Cefoxitin > 8mg/L (R) Cefotaxime/clavulanic acid or ceftazidime/clavulanic acid synergy
4	Carbapenemases	Meropenem > 0.12mg/L (R)
5	Other phenotypes	Cefotaxime or ceftazidime > 1mg/L (R) Cefoxitin < 8mg/L (S) No cefotaxime/clavulanic acid or ceftazidime/clavulanic acid synergy Cefoxitin < 8mg/L (S) Cefotaxime and ceftazidime ≤ 1mg/L Cefoxitin > 8mg/L (R) Cefotaxime or ceftazidime > 1mg/L (R) Meropenem ≤ 0.12 mg/L (S) Ertapenem > 0.06 mg/L (R) Imipenem > 1 mg/L (R) Other combinations, not described in this table but showing a resistance to β -lactams or carbapenems.

2.1.4. Definition of multidrug resistance

The term multidrug resistance refers to isolates with a resistance profile including at least three classes of antimicrobials. This means, for example, that resistance to ciprofloxacin and nalidxic 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 2022 than in previous years.

2.2. METHODOLOGY FOR METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

2.2.1. Sampling

In 2022, samples were taken of sows and fattening pigs. They are programmed to be taken by official veterinarians, distributed over the year and over the different local control units based respectively on the number of such farms in each control unit. Ten nasal swabs from 10 different fattening pigs or sows are taken on each holding and pooled to one sample. Samples of fattening pigs and sows may not be taken on a same holding. 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 herd is positive when MRSA is detected and confirmed by PCR.

2.2.2. Isolation and identification

Following the so-called 2-S isolation method used until mid-May 2022, pooled samples are incubated in Mueller-Hinton (MH) broth (Becton Dickinson) supplemented with NaCl (6.5%) at 37°C for 18-24h. One ml of this broth is added to Tryptic Soy Broth (TSB) supplemented with cefoxitin (3.5 mg/l) and aztreonam (75 mg/l) and incubated at 37°C for 18-24h. Ten microliter of this enrichment is plated on Brilliance MRSA 2 (Oxoid) and incubated 18-24h at 37°C.
From mid-May 2022, 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), in which the second enrichment step with cefoxitin and aztreonam is excluded.

2.2.3. Confirmation by real-time PCR

Presence of MRSA is suspected based on colony morphology. Per sample, one to five suspected colonies are selected from the Brilliance MRSA 2 plate. Presence of MRSA is confirmed using a triplex real-time PCR method. DNA is extracted using the DNeasy® Blood and Tissue kit according to the manufacturer instructions for Gram-positive bacteria (Qiagen, Hilden, Germany). The PCR allows detecting the *Staphylococcus aureus* specific gene, *nuc*, the presence of the *mec*A gene responsible for methicillin resistance and the variant *mec*C gene.

2.2.4. NGS

In 2022, a selection of 170 MRSA isolates were analyzed by NGS to determine their genotypes (sequence-types (ST) and *spa*-types) and also to detect their AMR and virulence genes. Thus, the antimicrobial resistance of MRSA in 2022 was only studied genetically and no more phenotypically. Genomic DNA was extracted as described above (section 2.5.3). 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 raw data was analyzed within the Veterinary Bacteriology department of Sciensano via the use of the *Staphylococcus* v0.1 pipeline, developed by the TAG

(Transversal activities in Applied Genomics) department of Sciensano and accessible on Galaxy (https://galaxy-tag.sciensano.be/). Use of the *Staphylococcus* pipeline includes rough fragment end trimming (Trimmomatic), assembly (SPAdes), MLST typing, spa typing, SCCmec cassette typing, detection of resistance genes, detection of mutations (PointFinder) and detection of virulence genes (VirulenceFinder). 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 *spa* gene encoding for the staphylococcal protein A is composed of repetitive regions. This method depicts the rapid evolution, since through recombination, the repeats may change fast. The "cgMLST scheme" tool published on PubMLST.org and the MLST Phylogeny v0.1 tool were used to investigate further the closeness of some isolates. Detection of antimicrobial resistance genes was performed as described by Bogaerts *et al.* (2021) and is based on the use of 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)

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 **Error! Reference source not found.**). 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. NGS

In 2022, a total of 19 enterococci were analyzed by NGS. 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* v0.1 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 *Escherichia coli* (*E. coli*), Methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis* and *Enterococcus faecium* indicator commensals in 2022 are presented below : monitoring in food products, 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).

Table 9. Number of isolates tested for antimicrobial resistance per matrix in 2022 (food)

Matrix	Number of isolates tested for MIC
Salmonella FOOD	
MIC 1st panel	46
MIC 2nd panel	1
Salmonella FEED	
MIC 1st panel	34
MIC 2nd panel	0
E. coli ESBL fresh beef meat (distribution)	
MIC 1st panel	5
MIC 2nd panel	5
E. coli ESBL fresh pig meat (distribution)	
MIC 1st panel	5
MIC 2nd panel	5
E. coli ESBL fresh turkey meat (distribution)	
MIC 1st panel	36
MIC 2nd panel	36
E. coli ESBL fresh broiler meat (distribution)	
MIC 1st panel	182
MIC 2nd panel	182
E. coli ESBL vegetables (distribution)	
MIC 1st panel	1
MIC 2nd panel	1
E. coli ESBL cow milk (farm)	
MIC 1st panel	33
MIC 2nd panel	33
Campylobacter FOOD	
MIC *C. jejuni*	46
MIC *C. coli*	23

3.1.1. Antimicrobial resistance in *Campylobacter* spp.

In 2022, the NRL received 74 presumptive *Campylobacter* spp. isolates from samples of poultry meat. The minimal inhibitory concentration (MIC) was determined on 69 isolates and the species identification was done by MALDI-TOF mass spectrometry and the results are shown in Table 10.

Table 10. Species identification of *Campylobacter* spp. tested for AMR in 2022

Species	Number
Campylobacter coli	23
Campylobacter jejuni	46
Campylobacter lari	0
Total	**69**

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 2022 per matrix of origin.

Technical Sheet	Description	Number	
		C. jejuni	***C. coli***
TRA 100	Fresh meat from poultry with skin	38	21
DIS 801	Mixed meat products	0	1
DIS 819	Fresh meat from broilers	6	1
DIS 821	Meat preparation from broilers	2	0

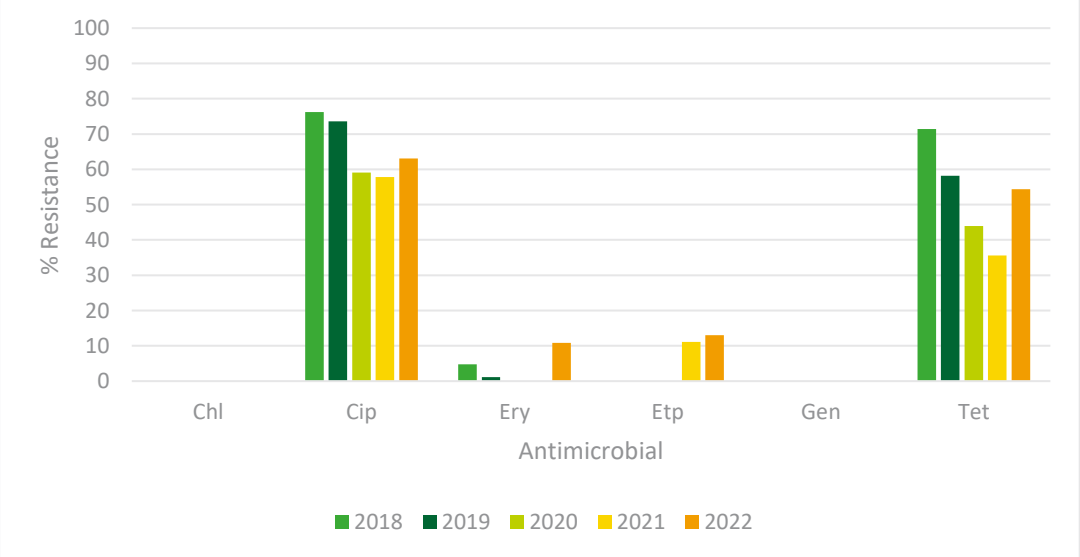


Figure 1. Trends in antimicrobial resistance rates in *C. jejuni* isolated from poultry meat (2018-2022).

In comparison with 2021, the levels of resistance in *C. jejuni* increased for all antimicrobials except for gentamicin and chloramphenicol to which resistance remains undected. Of particular interest in 2022, the end of the decreasing trend of resistance to tetracyline and ciprofloxacin and the moderate level of resistance to erythromycin which was not found in 2021 (Figure 1).

The number of isolates susceptible to every antimicrobial tested keeps decreasing since 2020. Multidrug resistance increased in 2022 after a decrease in 2021. 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 7 isolates were multidrug resistant compared to the 2 MDR isolates in 2021. 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 was added. Resistance to ertapenem was found in *Campylobacter* spp. isolated from various matrices since 2021.

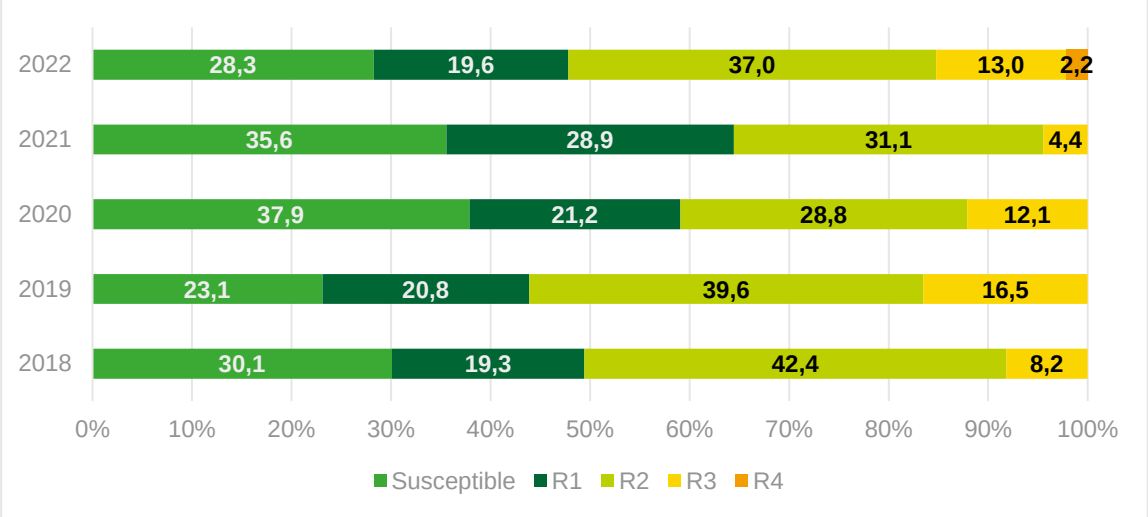


Figure 2. Percentages of susceptibility and resistance to one or more antimicrobial in *C. jejuni* (2018-2022)

In *Campylobacter coli* isolated from poultry meat in 2022, we notice an increase in resistance to ciprofloxacin reaching an extremely high level (>90%) (Figure 3). The extremely high resistance level detected in 2022 compared to 2021 could be associated to closely related isolates dominating in the poultry sector presenting a common resistance profile. On the other hand, resistance to erythromycin, ertapenem and tetracycline slightly decreased in 2022. Regarding the high resistance to ertapenem, it should be taken into account that there are no validation thresholds 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 in analysis.

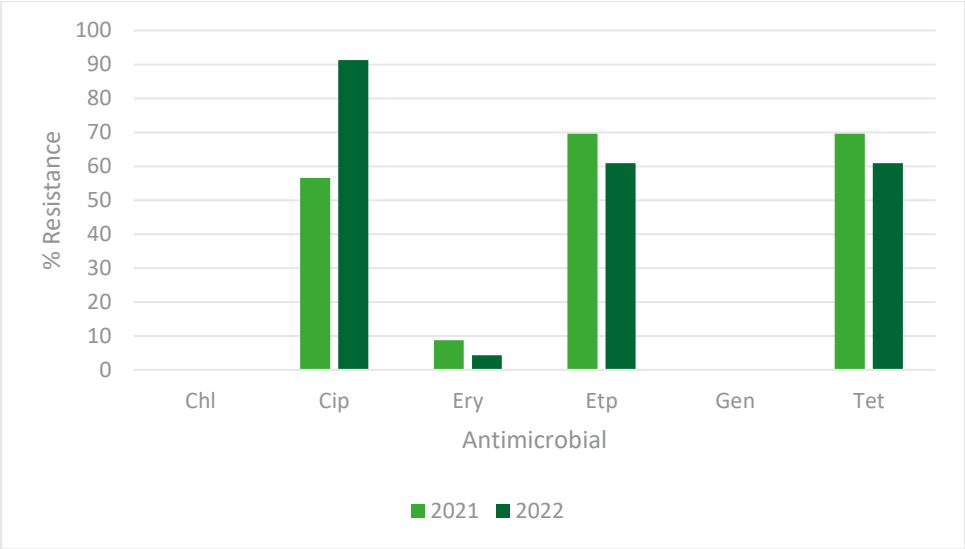


Figure 3. Trends in antimicrobial resistance rates in *C. coli* isolated from poultry meat (2021-2022)

The comparison of the resistance levels between *C. jejuni* and *C. coli* in 2022 shows that, as expected, overall resistances are higher in *C. coli* with the exception of erythromycin. The difference is most noticeable in the case of resistance to ertapenem and, to a lesser extent, tetracycline. Resistance to chloramphenicol or gentamicin has not been detected in either *C. jejuni* or *C. coli* isolated from poultry meat samples.

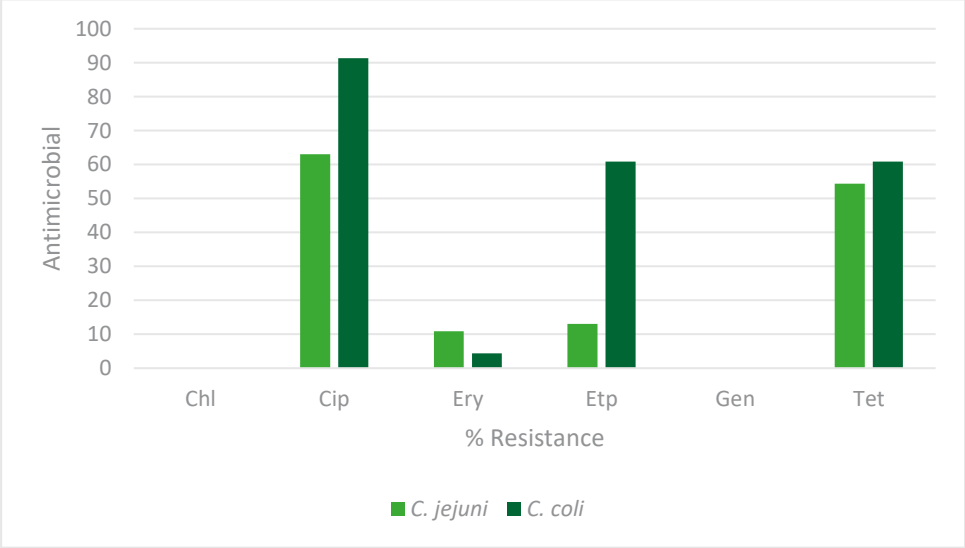


Figure 4. Levels of antimicrobial resistance in *C. jejuni* and *C. coli* isolated from poultry meat in 2022

3.1.2. Antimicrobial resistance in *Salmonella*

This section contains the analyses of the Salmonella food and feed programmes.

Since 2021, the Sensititre EUVSEC3 plates (Commission Implementing Decision 2020/1729/EU, Section 2, Table 3) are used to determine the MICs of the strains. Isolates showing resistance to either, cefotaxime, ceftazidime or meropenem were also tested for their ESBL phenotype using the EUVSEC2 Sensititre plate.

3.1.2.1. *Salmonella* FOOD

As part of the *Salmonella* FOOD programme, 46 isolates were analysed by antimicrobial susceptibility testing in 2022.

Figure 5 shows the percentage of isolates per serotype in food matrices. As in previous years, the most prevalent serotype is Infantis (55%) followed by Paratyphi B Var. L(+) Tartrate+ (18%).

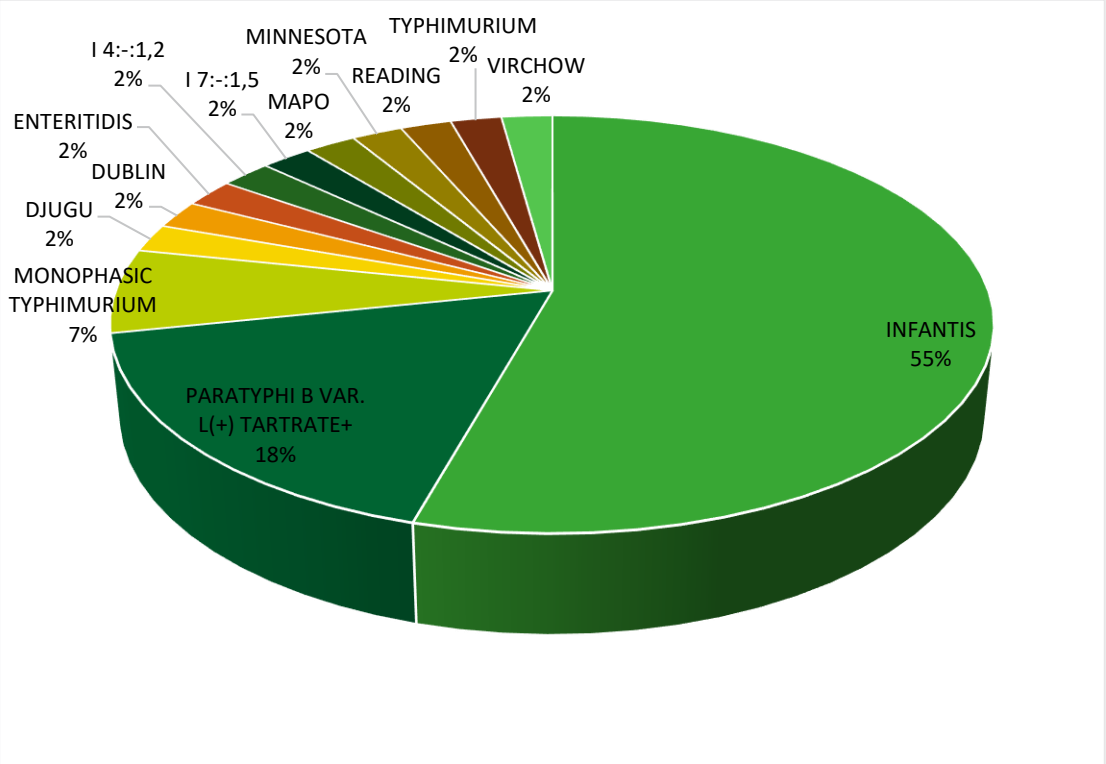


Figure 5. Percentage of *Salmonella* isolates per serotype in food matrices (n=46) in 2022.

Table 12 shows the number of *Salmonella* isolates per serotype and per food matrix.

Table 12. Number of Salmonella isolates per serotype and per food matrix in 2022.

Serotype/matrix	n
DJUGU	**1**
Cumin	1
DUBLIN	**1**
Fresh meat from poultry with skin	1
ENTERITIDIS	**1**
Fresh meat from poultry with skin	1
I 4:-:1,2	**1**
Fresh meat from poultry with skin	1
I 7:-:1,5	**1**
Meat preparation from bovine animals and pigs	1
INFANTIS	**25**
Crustaceans	1
Fresh meat from broilers	2
Fresh meat from poultry with skin	17
Meat preparation from bovine animals and pigs	3
Meat preparation from broilers	1
Meat preparation from poultry	1

MAPO	**1**
Fresh meat from poultry with skin	1
MINNESOTA	**1**
Fresh meat from poultry with skin	1
MONOPHASIC TYPHIMURIUM	**3**
Meat preparation from bovine animals and pigs	1
Meat preparation from pigs	1
Unspecified meat product	1
PARATYPHI B VAR. L(+) TARTRATE+	**8**
Fresh meat from poultry with skin	6
Meat preparation from bovine animals and pigs	1
Meat preparation from broilers	1
READING	**1**
Cumin	1
TYPHIMURIUM	**1**
Fresh meat from poultry with skin	1
VIRCHOW	**1**
Fresh meat from poultry with skin	1
Total	**46**

Figure 6 shows the antimicrobial resistance levels in all the *Salmonella* isolates (n=46) analysed in 2022 as part of the *Salmonella* Food programme.

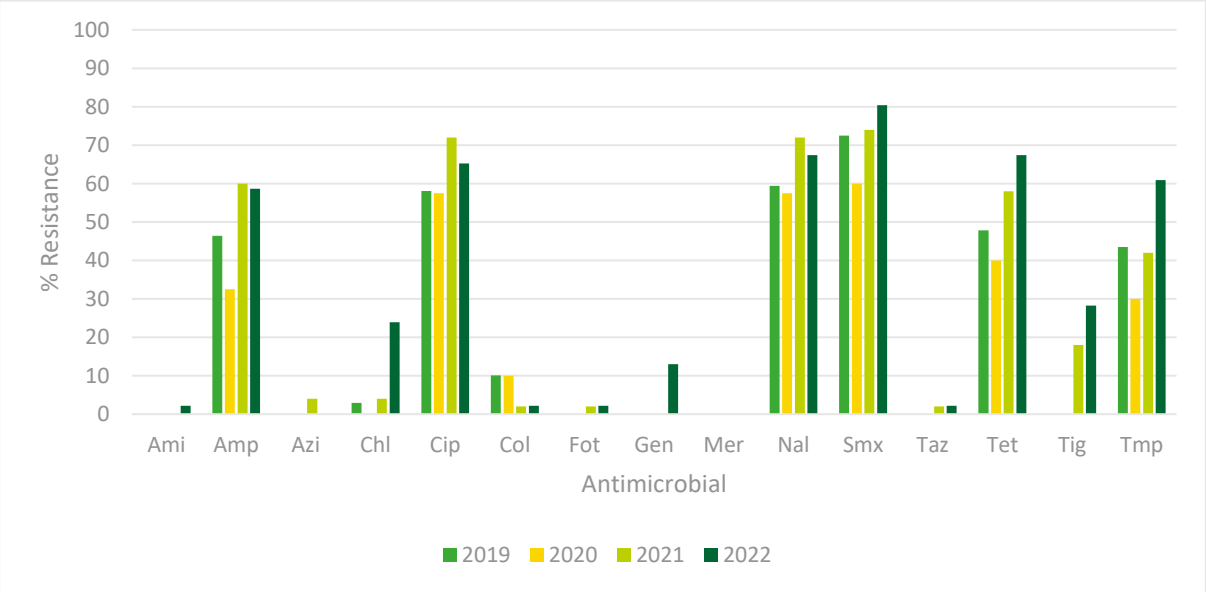


Figure 6. Trends in antimicrobial resistance rates in *Salmonella* spp. isolated from food (2019-2022)

Overall, in 2022, resistance rates to several antimicroials increased in comparison with 2021 when the trends already showed increases. Thus, resistance to chloramphenicol, sulfamethoxazole, tigecycline, and trimethoprim continued to increase, while moderate resistance to gentamicin and low resistance to amikacin were detected for the first time in 2022.

Resistance to 3rd generation cephalosporins was found in one out of 46 isolates tested that belonged to the serovar Paratyphi B var. L(+) Tartrate +. The level of resistance to colistin was very low, it was observed in only one isolate of *S*. Dublin. Resistance to tigecylcine was observed in 13 out of 46 isolates, however the MIC were only one dilution over the cut-off value (MIC 1 mg/L), this deviation is accepted as a limitation of the micro broth dilution method. Ths low level of resistance is not often associated to

plasmid-mediated tigecycline resistance genes. Confirmation of the resistance to tigecycline should be done by molecular methods.
Resistance to gentamicin was found in 6 isolates from 5 different serovars, *S*. Infantis (n=2), Typhimurium (n=1), Monophasic Typhimurium (n=1), Paratyphi B var. L(+) Tartrate + (n=1) and Subspi (n=1)
Comparison of trends with previous years has to be done with caution, since resistance is frequently associated to the serovar and the number of isolates per serovar per year may vary.
S. Infantis was the most predominant serovar in 2022, all but two isolates were resistant to ciprofloxacin, and all except one were resistant to sulfamethoxazole and tetracycline. Although a wide range of different MDR patterns were reported among *S*. Infantis isolates from food matrices, the most frequent core MDR pattern was AmpCipNalSmxTetTmp. Patterns of resistance associated with this serovars have a marked influence on the overall resistance levels in *Salmonella* spp.

3.1.2.2. *Salmonella* Feed

Figure 7 shows the percentage of isolates (n=34) per serotype in feed. The most prevalent serotype is Subspi (23%) followed by Infantis (20%) and Livinsgtone (18%).

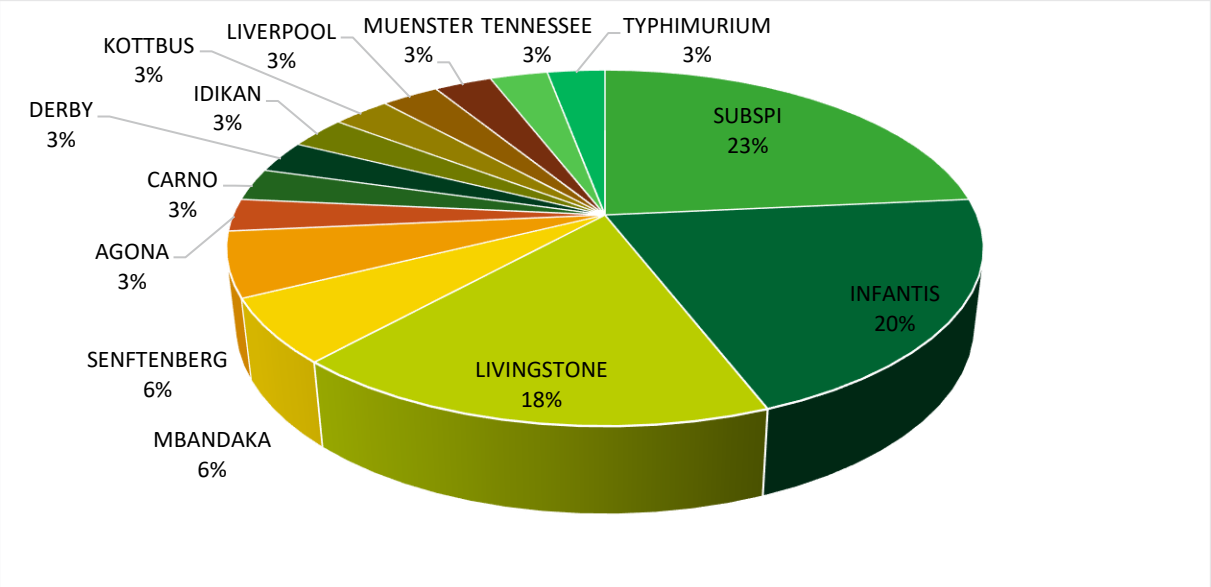


Figure 7. Percentage of serotypes of Salmonella spp. in feed matrices (n=34) in 2022

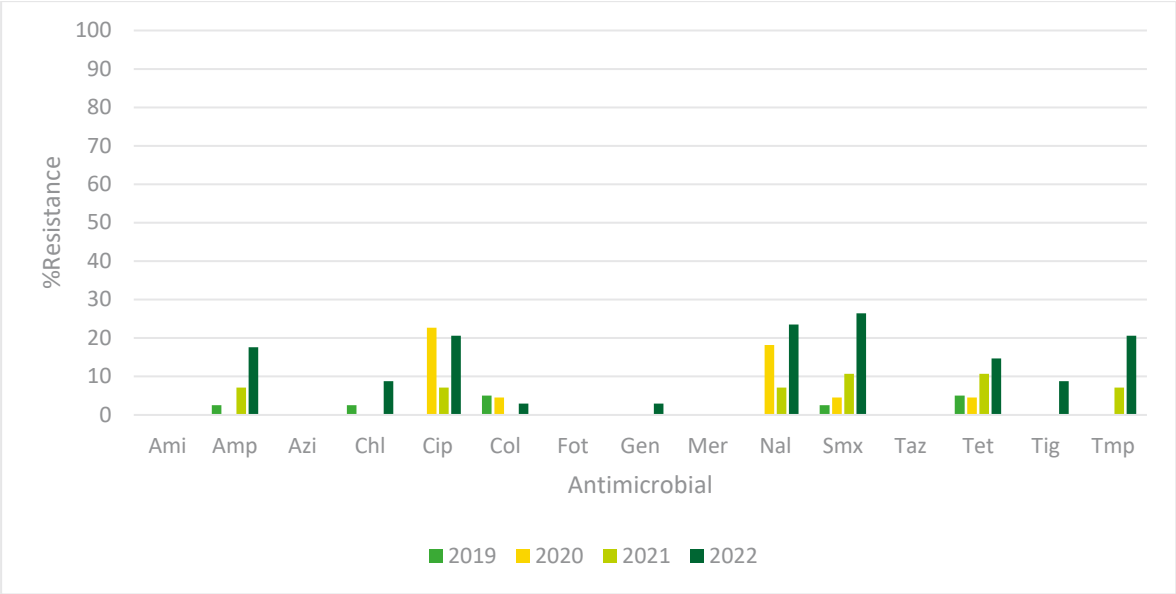


Figure 8. Trends in antimicrobial resistance rates in Salmonella spp. isolated from animal feed (2019-2022)

34 *Salmonella* spp. isolated from animal feed were subjected to antimicrobial susceptibility testing (Figure 8).
Antimicrobial resistance rates increased in 2022 for ampicillin, chloramphenicol, sulfamethoxazole, tetracycline and trimethoprim. Resistance to tigecycline was also found for the first time in 2022 for this matrix. (Fluoro)quinolone resistance decreased in 2021 but increased again in 2022 to reach levels similar to those in 2020. None of the isolates were resistant either to 3rd generation cephalosporins, amikacin or azithromycine.

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), but also samples from raw milk (PRI 013) and from vegetables (DIS 841) were tested for the detection of ESBL *E.coli*.
Since 2014, a specific monitoring is performed : all isolates that show resistance to third generation cephalosporins and are presumptive ESBL *E.coli* are analysed through the first and second panel of antimicrobials as indicated in section 2, tables 5 and 6.
Given the low prevalence of ESBL *E. coli* in fresh pig meat and bovine meat (DIS 809) and in vegetables (DIS 841) and therefore the low number of isolates analysed for these matrices, confidence intervals will not be presented as they are not relevant.

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

In 2022, as part of the specific search for *E. coli* producing ESBL, AmpC or carbapenemases 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 *E. coli* ESBL 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), 52 (50%) and 130 (62,5%) respectively were positive for the detection of ESBL

E.coli. Hence, the prevalence of ESBL *E. coli* in broiler meat in 2022 is 58,33% compared to 56% in 2021. A gradual decrease of the prevalence of *E.coli* ESBL/AmpC has been noticed over the years. From 80% reported in 2016 to 58,33% in 2022. A presumptive carbapenemase producing *E. coli* was also isolated but antimicrobial susceptibility testing did not confirm the carbapenemase phenotype. Antimicrobial susceptibility testing was performed on all 182 isolates (Figure 9).

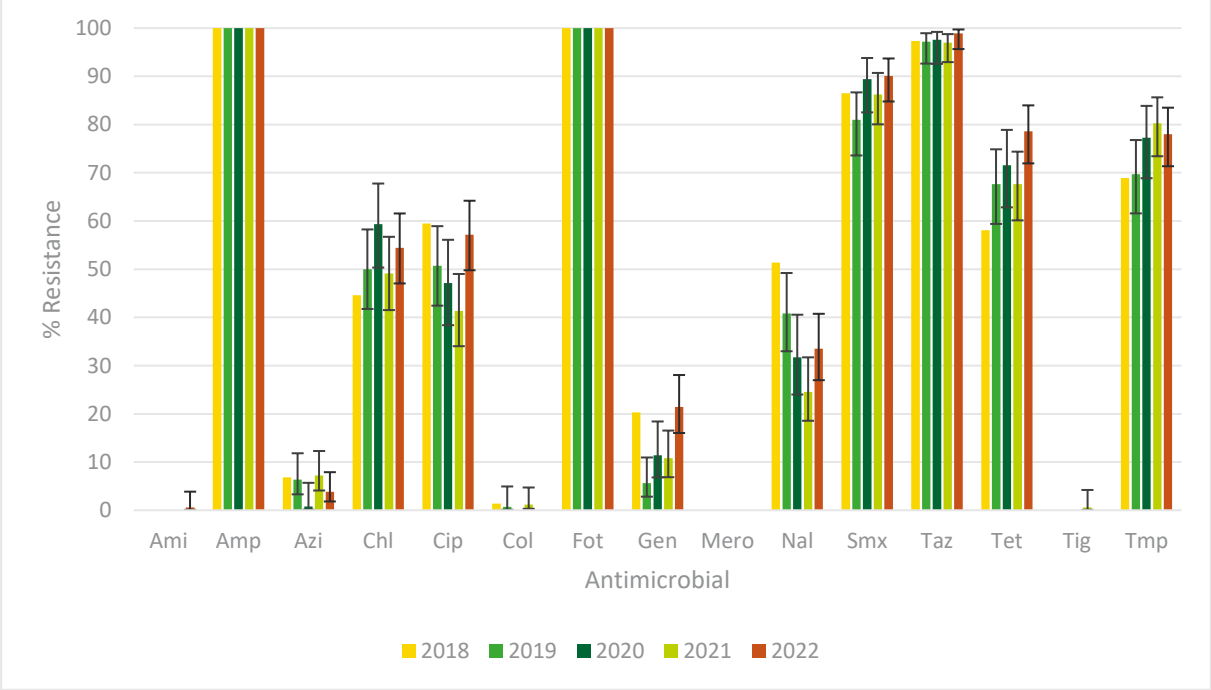


Figure 9. Trends in resistance rates to the first panel of antimicrobials in ESBL *E.coli* isolated from broiler meat (2018-2022)

As expected since the isolates come from the specific monitoring of ESBL *E.coli*, resistances to 3rd generation cephalosporins and ampicillin are extremely high in 2022 and all the isolates were confirmed to be β-lactamase producers. Resistance to tetracycline also increased from very high in 2021 to extremely high in 2022. However the only significant change in comparison with 2021 is the increase of resistance to ciprofloxacin from a high (41,32%) to a very high (57,14%) level. The resistance to ciprofloxacin was accompanied by resistance to sulfamethoxazole, tetracycline and trimethoprim. Molecular investigations would be needed to show whether the isolates carry any transferable genes encoding for ciprofloxacin resistance in addition to β-lactamases. The resistance level to gentamicin also significantly increased from 2019 to 2022. Amikacin resistance (monitored since 2021) was also detected in one isolate for the first time in broiler meat in 2022. However, resistances to colistin and tigecycline were not detected in 2022 in contrast to 2021. Resistance to meropenem was also not detected in 2022.

The percentage of isolates displaying a multidrug resistant phenotype accounted to 96.70%.

Any *E. coli* isolate showing resistance to cefotaxime, ceftazidime or meropenem had to be subjected to the second panel analysis (EUVSEC2) and interpreted according to Table 6. The second panel allows a precise categorisation of *E. coli* isolates showing resistance to 3rd generation cephalosporins. According to this categorisation, 95% of the isolates had an ESBL phenotype, 3% had an AmpC phenotype, and 2% had a phenotype that was a combination of ESBL + AmpC (Figure 10).

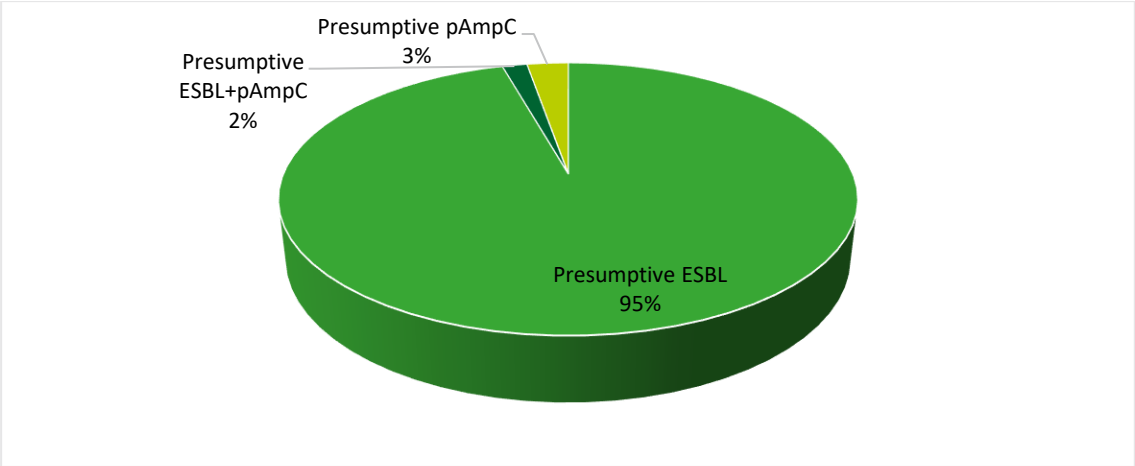


Figure 10. Percentage of phenotypes obtained using the second panel of antimicrobials in ESBL *E. coli* isolated from poultry meat (DIS 819 and DIS 821) in 2022

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.

311 samples of bovine meat, 314 samples of pig meat and 156 samples of turkey meat were tested for the detection of ESBL *E.coli* and 6 (1,93%), 5 (1,59%) and 37 (23,7%) samples were positive respectively.

The percentages of resistance to the first panel of antibiotics are shown in Figure 11 for bovine meat and in Figure 12 and Figure 13 for pig and turkey meat.

Figure 11. Trends in resistance rates to the first panel of antimicrobials in ESBL *E.coli* isolated from bovine meat (2018-2022).

Given the very low prevalence of ESBL *E. coli* in bovine and pig meat, the interpretation of the trends in resistance should be undertaken with caution. Overall, the resistance profiles seem stable from one year to another. We can note however that in 2022, the 5 isolates tested for their antimicrobial susceptibility were resistant to tetracycline. One isolate was also resistant to colistin, confirming the appearance of this resistance detected in 2021 for this matrix. A common core resistant pattern of Amp-Fot-Taz-Chl-Cip-Tet-Tmp-Smx was found in 3 out of 5 isolates. No meropenem or amikacin resistance was detected in 2022. All 5 isolates had an ESBL phenotype.

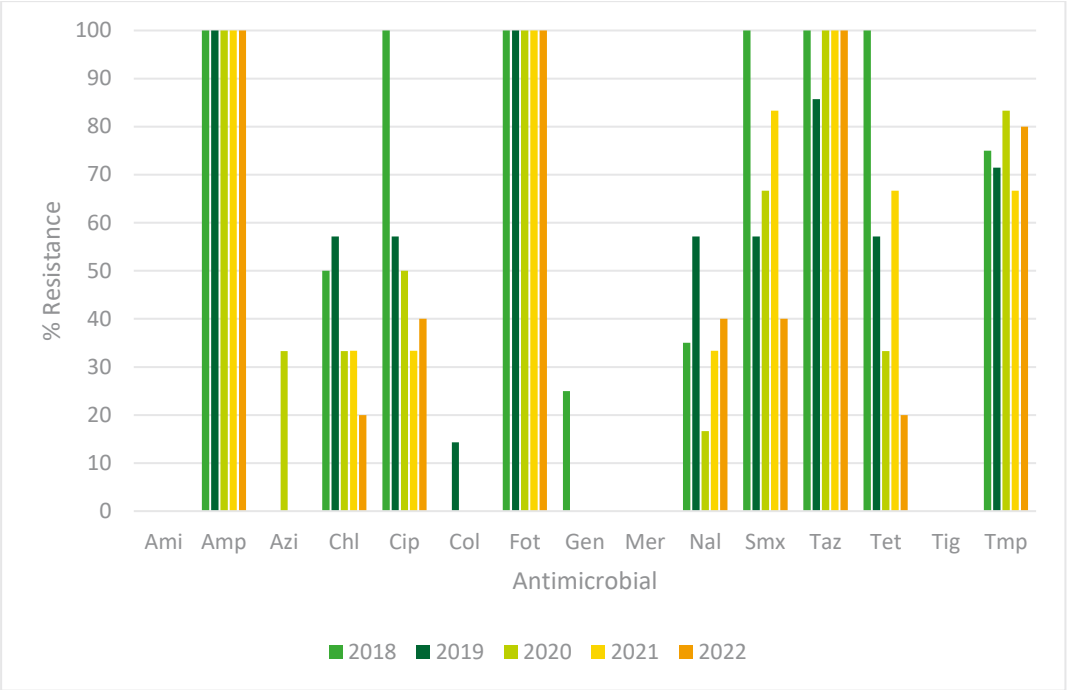


Figure 12. Trends in resistance rates to the first panel of antimicrobials in ESBL *E.coli* isolated from pig meat (2018-2022).

As mentioned for the isolates sampled from bovine meat, the very low prevalence of ESBL *E.coli* in pig meat affects the interpretation of trends. In 2022, in the 5 ESBL *E. coli* isolates tested, no resistance to amikacin, azithromycin, colistin, gentamicin, tigecycline or meropenem was detected (Figure 12). One isolate only showed the expected resistance to 3rd generation cephalosporins and ampicillin and was therefore not considered multidrug resistant. 4 of the 5 isolates had an ESBL phenotype and the last one had an AmpC phenotype.

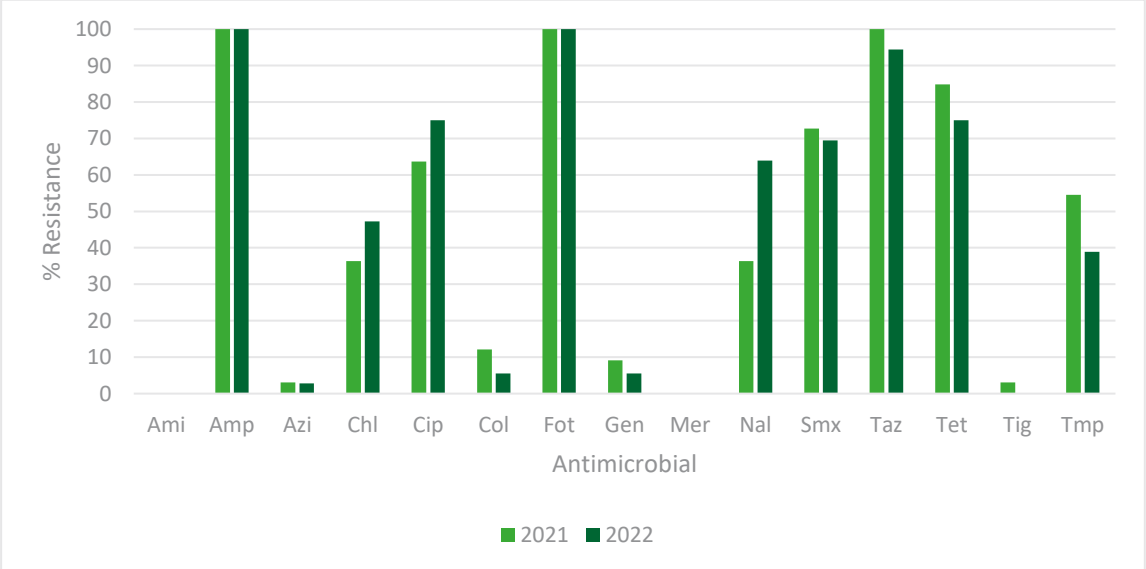


Figure 13. Trends in resistance rates to the first panel of antimicrobials in ESBL *E.coli* isolated from turkey meat (2021-2022).

In the 36 ESBL *E.coli* isolated from turkey meat tested for their susceptibility, the resistance rates decreased or were stable for most antibiotics (Figure 13). However, resistance to (fluoro)quinolones, often detected in poultry matrices, slightly increased since 2021. 31 isolates (86,11%) were multidrug resistant. Testing of the second panel of antimicrobials allowed for the clasification of the isolates

according to their phenotype. 78% had an ESBL phenotype, 14% had an AmpC phenotype (Figure 14). One isolate was identified as a carbapenemase producing ESBL *E .coli* because it was resistant to ertapenem. However, no resistance to meropenem or imipenem was detected in the first or second panel. This isolate is therefore not listed as a carbapenemase producer according to EFSA criteria. Two isolates were classified as « other phenotypes » because they showed a resistance to 3rd generation cephalosporins without clavulanic acid synergy while they were sensitive to cefoxitin.

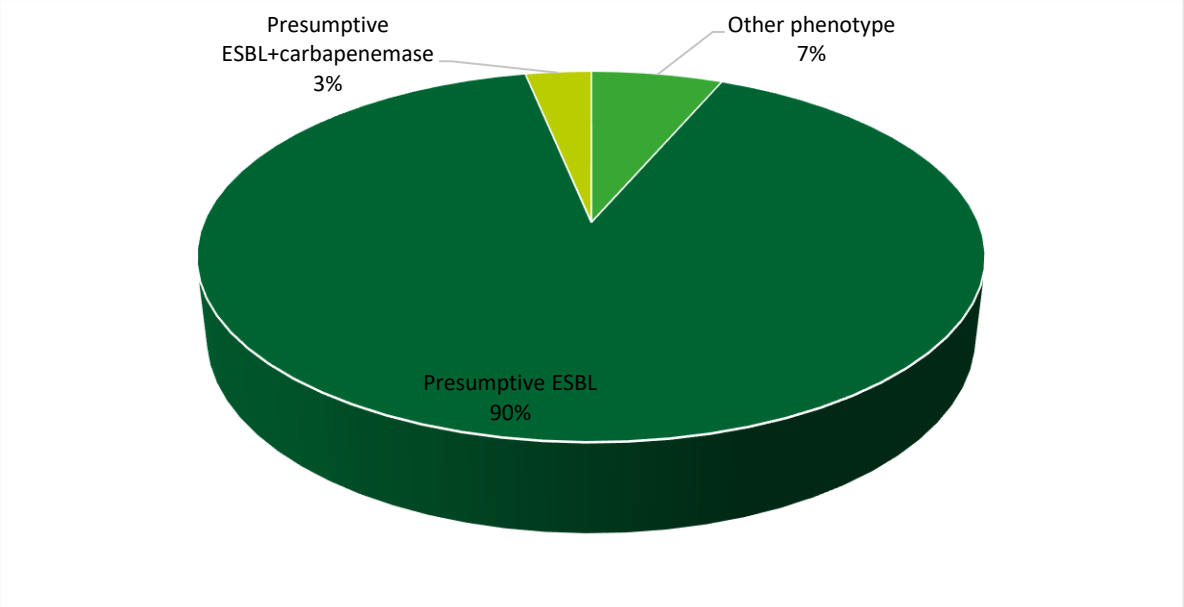


Figure 14. Percentage of phenotypes obtained using the second panel of antimicrobials in ESBL *E. coli* isolated from turkey meat (DIS 809) in 2022

3.1.3.2. Specific monitoring of ESBL, AmpC or carbapenemase producing *E.coli* in raw milk

As part of the specific search for ESBL, AmpC or carbapenemase producing *E. coli* in raw cow's milk (PRI 013), a qualitative method (detection/non-detection in 25ml) 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.

In 2022, 300 samples of raw milk (25ml) were tested for the detection of ESBL and AmpC *E. coli* and 34 were positive (11,33%). 33 of those were tested for their antimicrobial susceptibility.

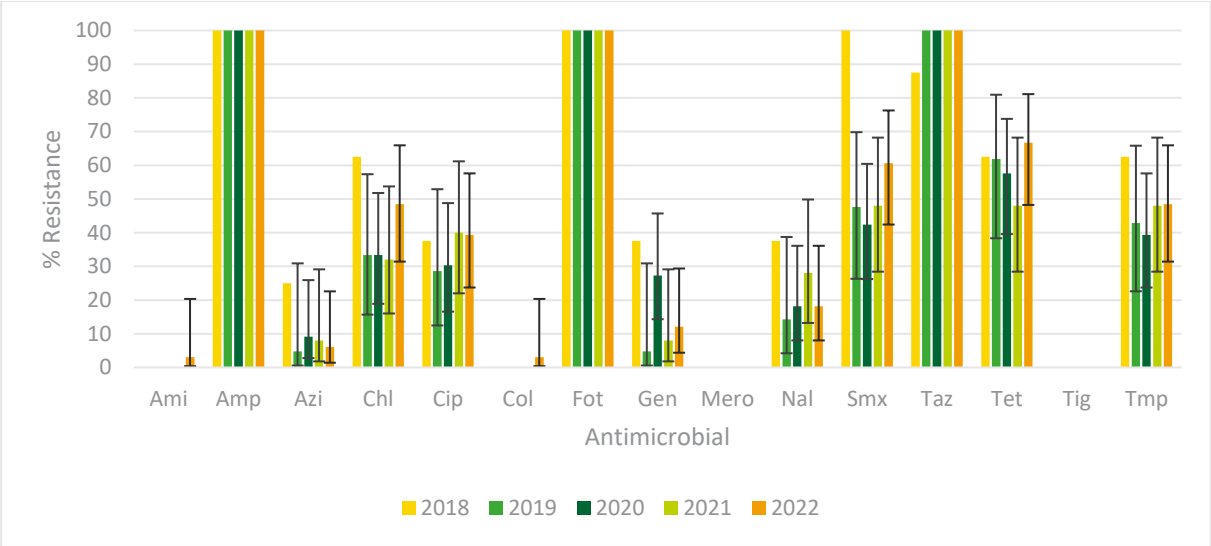


Figure 15. Trends in resistance rates to the first panel of antimicrobials in ESBL *E.coli* isolated from raw cow's milk (2018-2022)

There were no significant changes in resistance trends in 2022 compared to previous years, with the exception of one isolate showing resistance to colistin for the first time and another isolate showing resistance to amikacin, also for the first time in 2022. However, we can note the decrease in resistance to nalidixic acid in 2022 after an increase in 2021. We also note an increase in resistance to tetracycline whereas this resistance had been decreasing since 2018. 76% of the isolates were multidrug resistant and two isolates showed a resistance to nine different antimicrobial classes. The categorisation following the testing of the second panel of antimicrobials showed that 73% have an ESBL phenotype, 12% have an AmpC phenotype and 6% have a combination of both ESBL+AmpC phenotypes (Figure 16). Moreover, one isolate was identified as an ESBL with carbapenemase production because it was resistant to ertapenem. However, no resistance to meropenem or imipenem was detected in the first or second panel. This isolate is therefore not listed as a carbapenemase producer according to EFSA criteria. Also, two isolates were classified as « other phenotpyes » because they showed a resistance to 3rd generation cephalosporins and no clavulanic acid synergy while they were sensitive to cefoxitin.

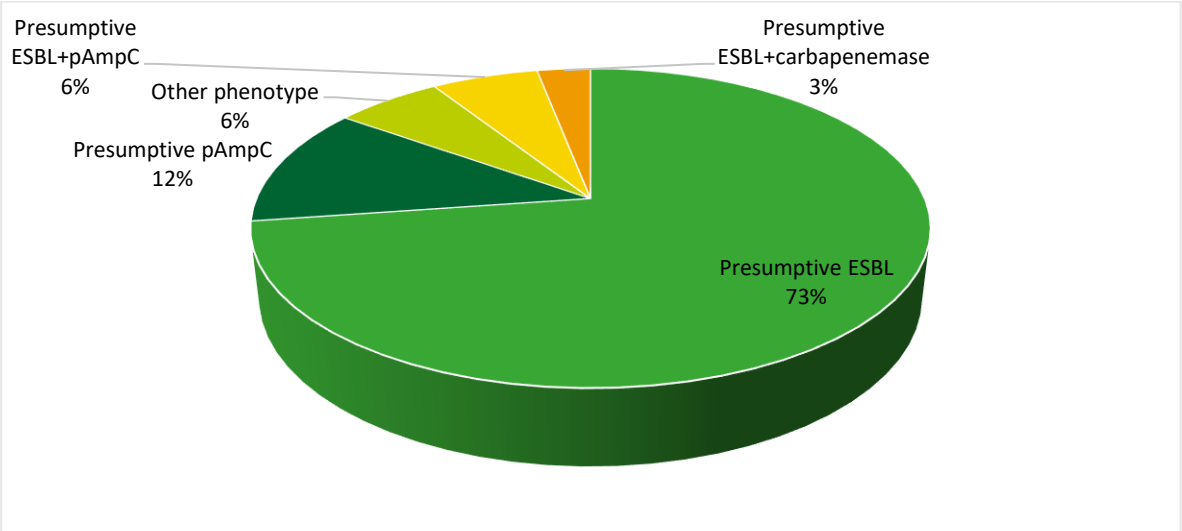


Figure 16. Percentage of phenotypes obtained using the second panel of antimicrobials in ESBL *E. coli* isolated from raw cow's milk (PRI 013) in 2022

3.1.3.3. Specific monitoring of ESBL, AmpC or carbapenemase producing *E.coli* in vegetables

As part of the specific search for ESBL, AmpC or carbapenemase producing E. coli in vegetables (DIS 841), 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.

In 2022, 293 samples of vegetables were tested for the detection of ESBL E.coli and only one was positive (0,3%). The isolate was detected in a sample of lettuce and was analysed by antimicrobial susceptibility testing. It showed resistance to ampicillin, cefotaxime and ceftazidime and had an ESBL phenotype.

3.2. ANTIMICROBIAL RESISTANCE MONITORING IN ZOONOTIC AND COMMENSAL BACTERIA ISOLOATED 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 2022, 301 samples of caeca from broilers were taken at the slaughterhouse for the detection of *Campylobacter* spp. and 113 were positive. Of those, 50 were identified as *C. jejuni* and 39 as *C. coli* by MALDI-TOF MS and were subjected to antimicrobial susceptibility testing by broth microdilution method. This monitoring was also conducted in previous years (2017-2018-2020) but only in *C. jejuni* and the antibiotics tested included streptomycin and nalidixic acid which are no longer tested and did not include chloramphenicol and ertapenem which are tested since 2021 according to the Commission Implementing Decision 2020/1729.

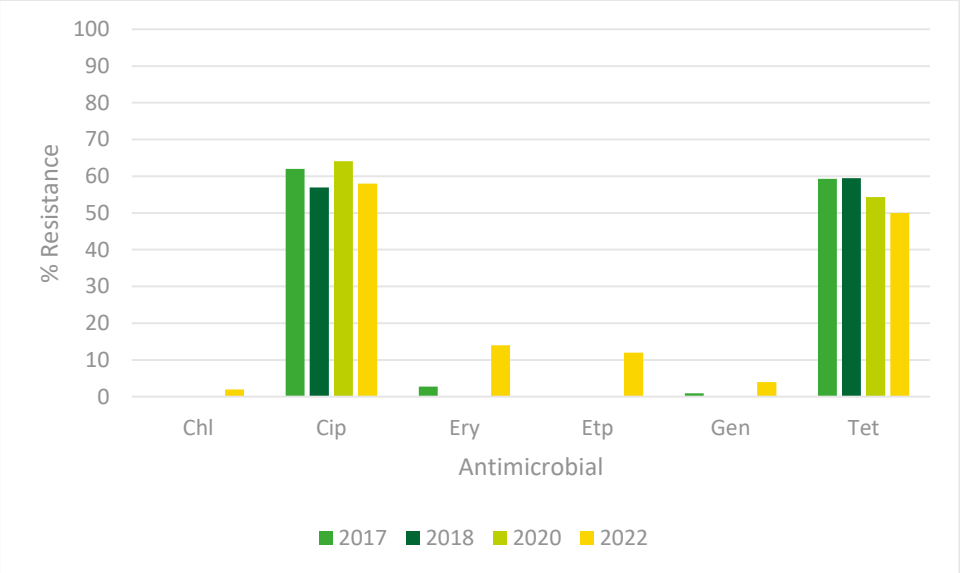


Figure 17. Trends in antimicrobial resistance rates in *C. jejuni* isolated from broiler caecal content

In comparison with previous years, resistance to erythromycin isolates and gentamicin increased in *C. jejuni* (Figure 17). Resistance to ciprofloxacin is stable and a decreasing resistance trend can be noted

for tetracycline. 12% of isolates were resistant to ertapenem and 2% were resistant to chloramphenicol, the antibiotics tested since 2021 for *Campylobacter* spp..
Combined resistance to both ciprofloxacin and erythromycin, which are considered critically important for treatment of campylobacteriosis, was moderate, 4 isolates out of 50 in *C. jejuni* from poultry displayed this resistance pattern.

In 2022 the monitoring was also conducted in 39 *Campylobacter coli* isolated from broiler caeca. Figure 18 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 tetracyclin and even more so for resistance to ertapenem. Combined resistance to ciprofloxacin and erythromycin was observed in 6 out of 39 isolates, representing 15.4% of the isolates of *C. coli* versus 8% in *C. jejuni*. 43,6% of *C. coli* isolates were multidrug resistant and none was susceptible to all the antimicrobial tested whereas 16% of *C. jejuni* isolates were multidrug resistant and 32% were susceptible to all the antimicrobial tested.

The most common pattern in both *C. jejuni* and *C. coli* was resistance to both ciprofloxacin and tetracycline, observed in 44% of *C. jejuni* isolates and 66.6 % 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 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 in analysis.

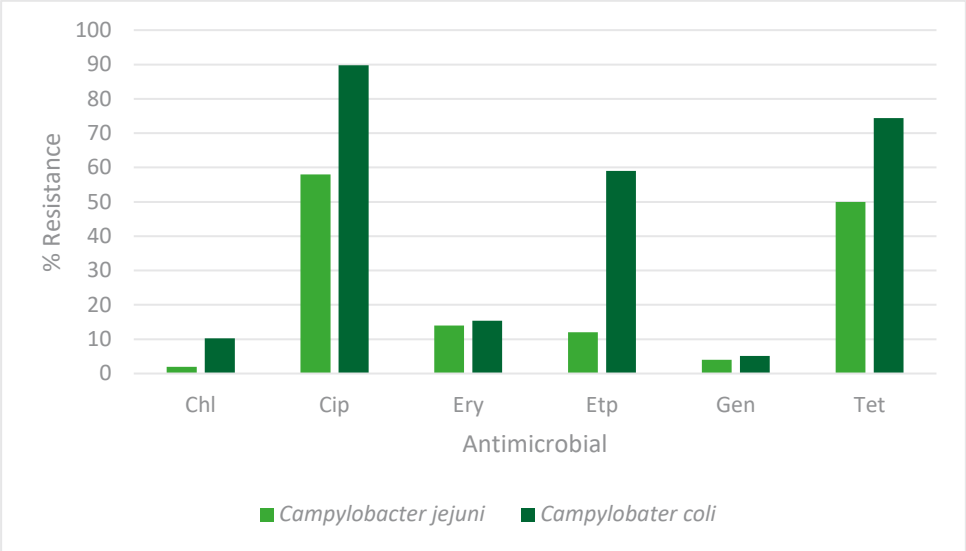


Figure 18. Comparison of resistance levels in *C. jejuni* and in *C. coli* isolated from broiler caecal content in 2022

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

In 2022, 300 samples of pig caeca taken at the slaughterhouse were tested for the detection of *Campylobacter coli* and 208 were positive. The minimal inhibitory concentration (MIC) was determined for 164 isolates (Figure 19).

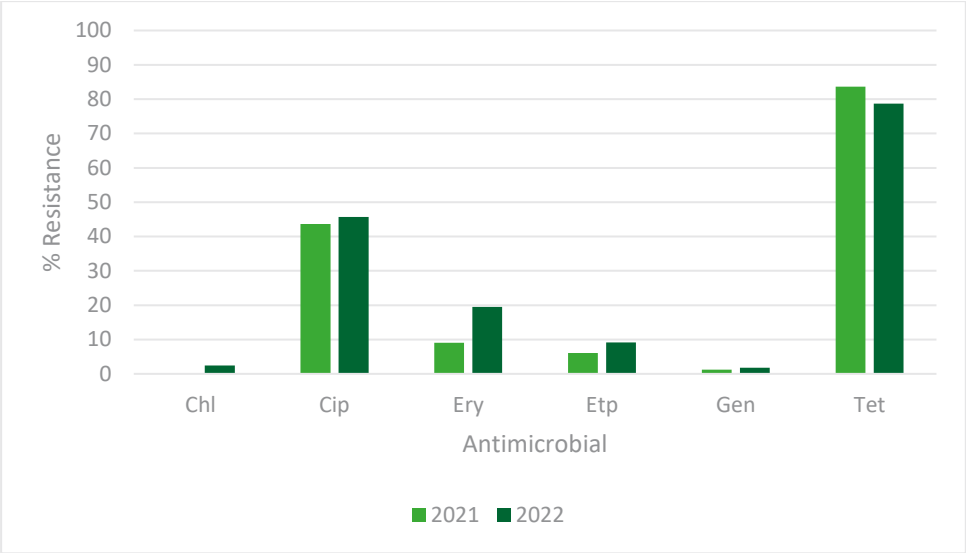


Figure 19. Trends in resistance rates in *C. coli* isolated from pig caecal content (2021-2022)

Figure 19 shows that resistance to erythromycin increased from low in 2021 to moderate in 2022. A low level of resistance to chloramphenicol was also detected in 2022 whereas it had not been detected in 2021. Other resistance levels remained stable in 2022. The most common core pattern of resistance included ciprofloxacin and tetracycline, 64 out ot 164 (39%) isolates were resistant to both antimicrobials. Combined resistance to critically important antimicrobials ciprofloxacin and erythromycin accounted for 14% (23 out of 164 isolates). Moreover the combined pattern CipEryTet was observed in 20 out of 164 isolates (12%). 15,85% of isolates were multidrug resistant and 13,41% were susceptible to all antimicrobials tested.

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

301 samples of bovine caecal content were taken at the slaughterhouse in 2022 for the detection of *Campylobacter* spp. isolates and 209 were positive. Of those, 135 *C. jejuni* and 74 *C. coli* were subjected to antimicrobial susceptibility testing.

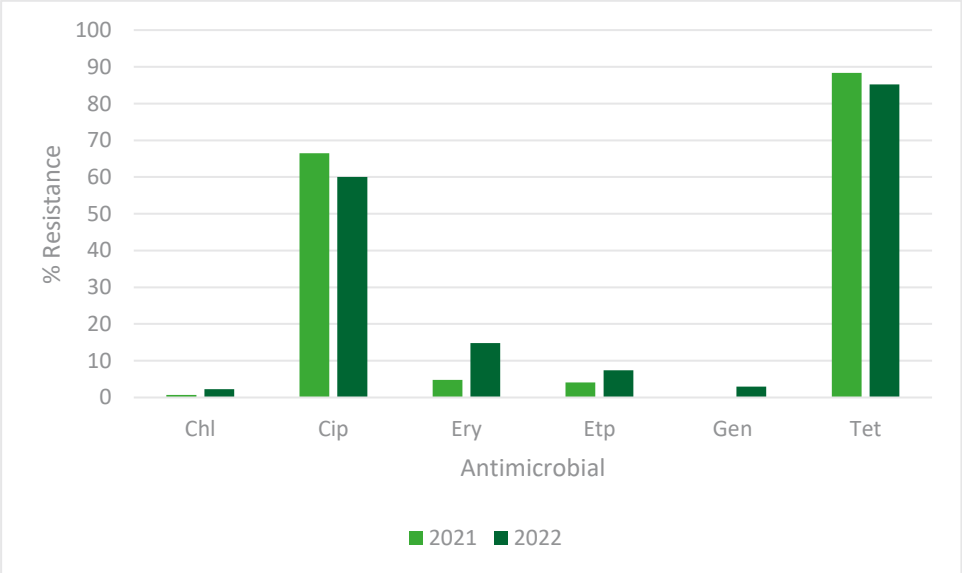


Figure 20. Trends in resistance rates in *C. jejuni* isolated from bovine caecal content (2021-2022)

In *Campylobacter jejuni*, resistances to chloramphenicol, erythromycin and ertapenem increased in 2022 and resistance to gentamicin was detected for the first time but resistance to ciprofloxacin and tetracycline slightly decreased (Figure 20).
The most common core pattern of resistance included ciprofloxacin and tetracycline, 75 out ot 135 (55.5%) isolates were resistant to both antimicrobials. Combined resistance to critically important antimicrobials ciprofloxacin and erythromycin accounted for 12.6% (17 out of 135 isolates). Moreover the combined pattern CipEryTet was observed in 17 out of 135 isolates (12.6%).

In 2022, 22 isolates of *C. jejuni* were multidrug resistant (16,30%) and 13 were susceptible to all antimicrobials tested (9,63%).

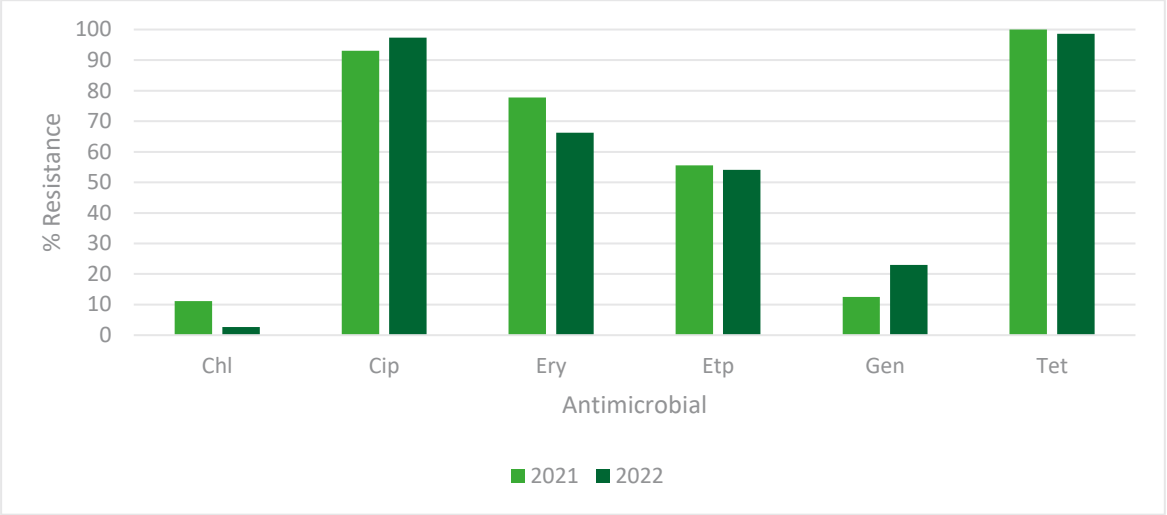


Figure 21. Trends in resistance rates in *C. coli* isolated from bovine caecal content (2021-2022)

However, in contrast to *Campylobacter jejuni*, Figure 21 shows that in *Campylobacter coli* resistance to chloramphenicol and erythromycin has decreased, but resistance to ciprofloxacin has increased. Resistance to gentamicin also increased and, as expected, the level of multidrug resistance was much higher in *C. coli* than in *C. jejuni* with 85,14% of *C. coli* isolates resistant to 3 or more classes of antibiotics. Moreover, only one isolate (1,35%) of *C. coli* was susceptible to all antimicrobials tested.

72 out of 74 isolates (97.3%) were resistant to ciprofloxacin and tetracycline. Combined resistance to critically important antimicrobials ciprofloxacin and erythromycin accounted for 66% of the isolates (49 out of 74). Moreover this pattern was accompanied by resistance to tetracycline as well (CipEryTet).

The difference in the levels of resistance is represented in Figure 22.

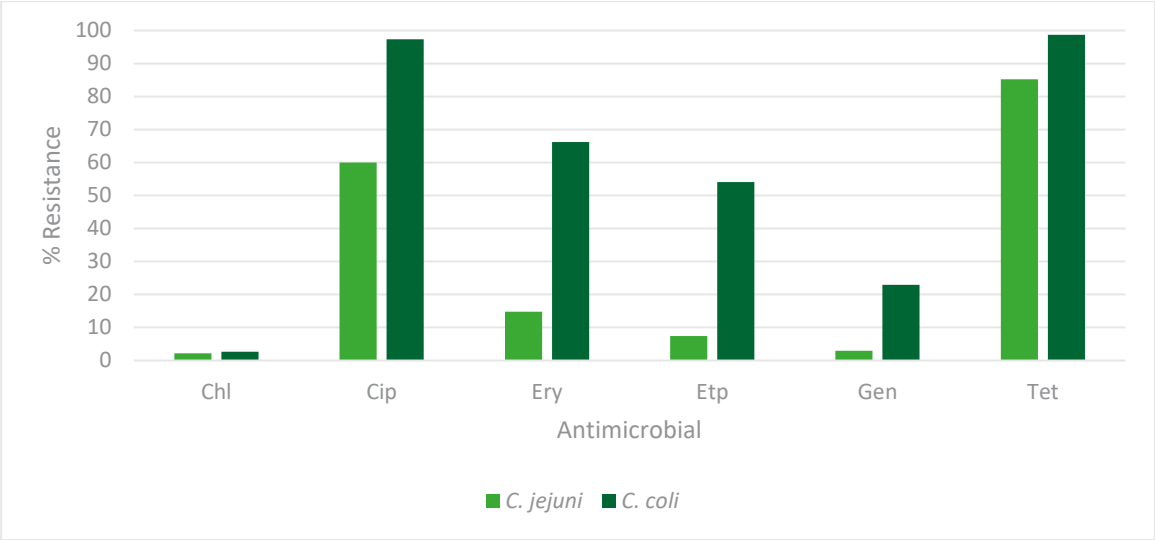


Figure 22. Comparison of resistance rates in *C. jejuni* and in *C. coli* isolated from bovine caecal content in 2022

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, 35 isolates were tested for antimicrobial susceptibility.

Figure 23 shows the percentage of *Salmonella* serotypes identified in pig caeca. The most prevalent serotype is monophasic Typhimurium (46%), followed by Derby (20%) and Typhimurium (20%)

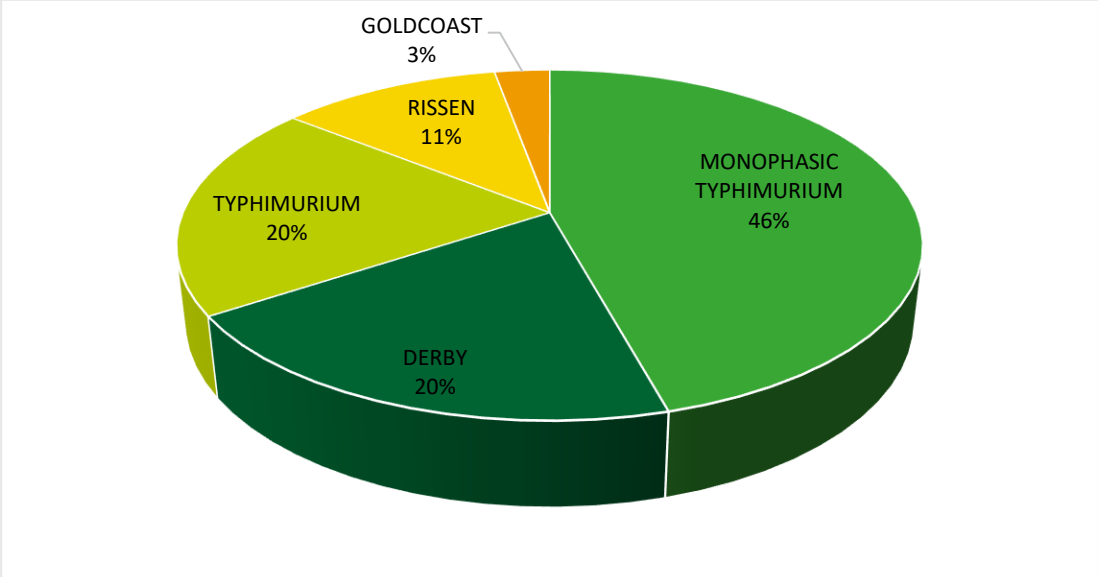


Figure 23. Percentage of serotypes identified for *Salmonella* isolated from pig caecal content

Figure 24 shows the resistance rate of all *Salmonella* spp. isolated from pig caeca and tested for antimicrobial susceptibility. Apart from a decreased resistance to tigecycline and no detection of resistance to colistin, in 2022 an increase in resistance to most antibiotics tested was observed in comparison with 2021. 51,43% of the isolates were multidrug resistant and 17,14% were susceptible to all antimicrobial tested. Considering the serotype Typhimurium and its monophasic variant, 91% of the isolates were resistant to ampicillin, followed by 65% to sulfamethoxazole and 60% to tetracycline. None of them were resistant to ciprofloxacin. Resistance to ciprofloxacin was observed in 3 isolates, belonging

to serotype Rissen (n=2) and Goldcoast (n=1). None of the isolates displayed resistance to 3rd generation cephalosporins. The Monophasic Typhimurium serotype contained the most multidrug resistant isolates while the Derby serotype contained the most fully susceptible isolates.

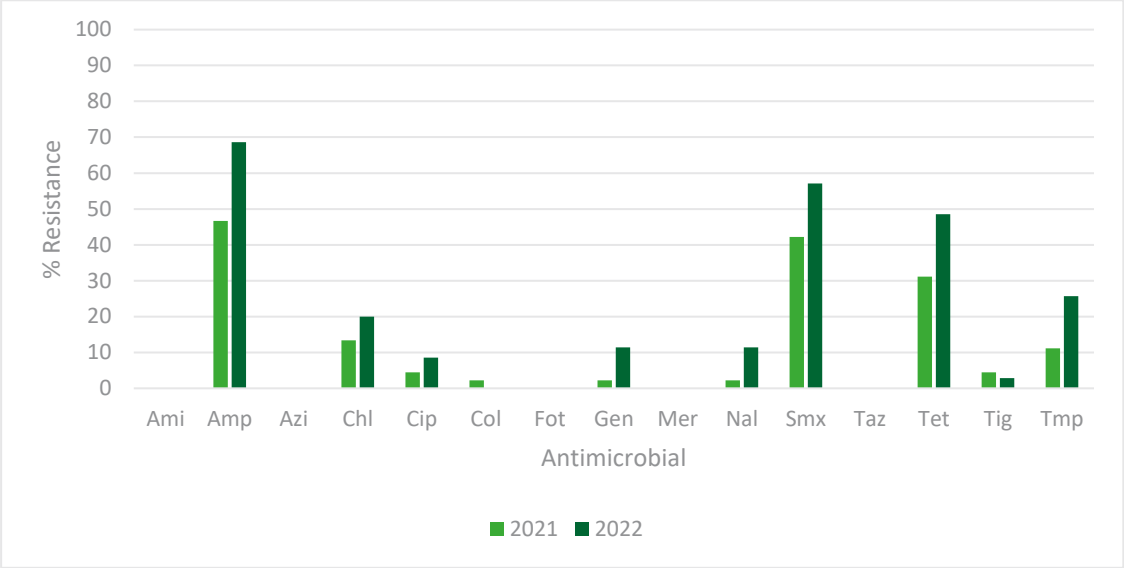


Figure 24. Trends in resistance rates in *Salmonella* spp. isolated from pig caecal content (2021-2022)

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

3 isolates were tested in 2022 as part of the monitoring of *Salmonella* spp. isolated from samples of bovine caeca collected at the slaughterhouse. The serotype identified were monophasic Typhimurium, Kottbus and Dublin. The isolates of *S*. monophasic Typhimurium and *S*. Dublin were multidrug resistant and *S*. Kottbus was only resistant to sulfamethoxazole. The *S.* Dublin isolate showed resistance to cefotaxime and was therefore tested with the second panel of antimicrobials which confirmed an ESBL phenotype.

3.2.6. Monitoring of antimicrobial resistance in *Salmonella* spp. isolated from poultry environmental samples

In 2022, environmental samples of broilers taken at the farm level three weeks before slaughter (Braad-uit) and of laying hens (Leg-Mon) were collected.

Out of 11076 environmental samples of broilers tested, 233 were positive for *Salmonella* spp. and 169 were tested for antimicrobial susceptibility.

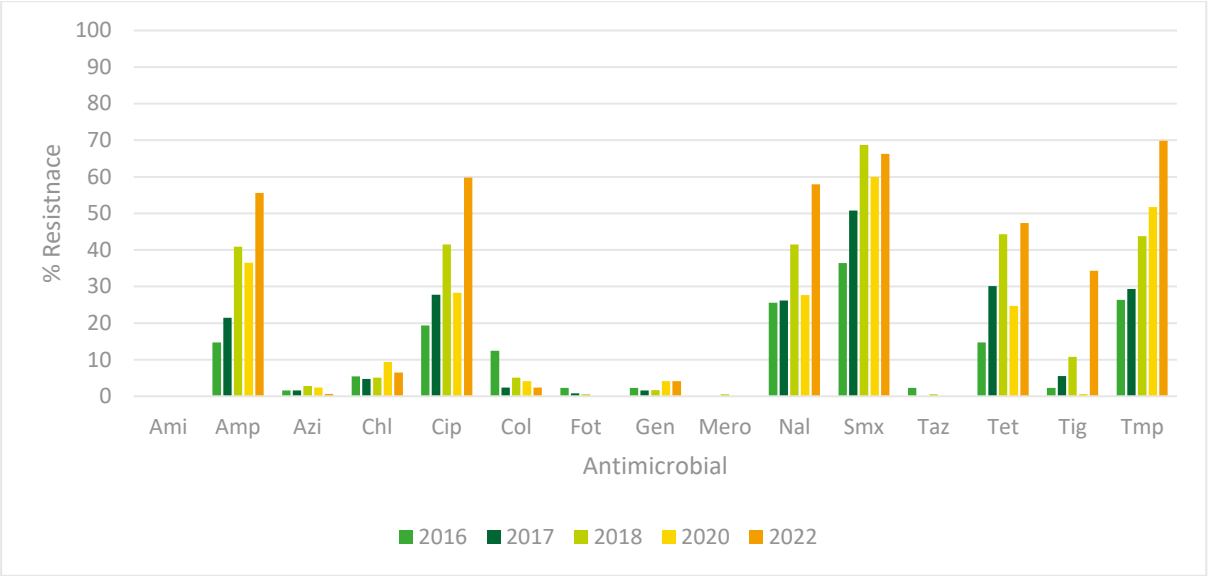


Figure 25. Trends in resistance rates in *Salmonella* spp. isolated from broilers environmental samples

Figure 25 shows that, in 2022, a strongly increased resistance to several antimicrobials was detected, especially to (fluoro)quinolones, ampicillin and tigecycline. We can also note an increasing resistance trend to trimethoprim. However, no resistance to amikacin, meropenem or 3rd generation cephalosporins was detected.

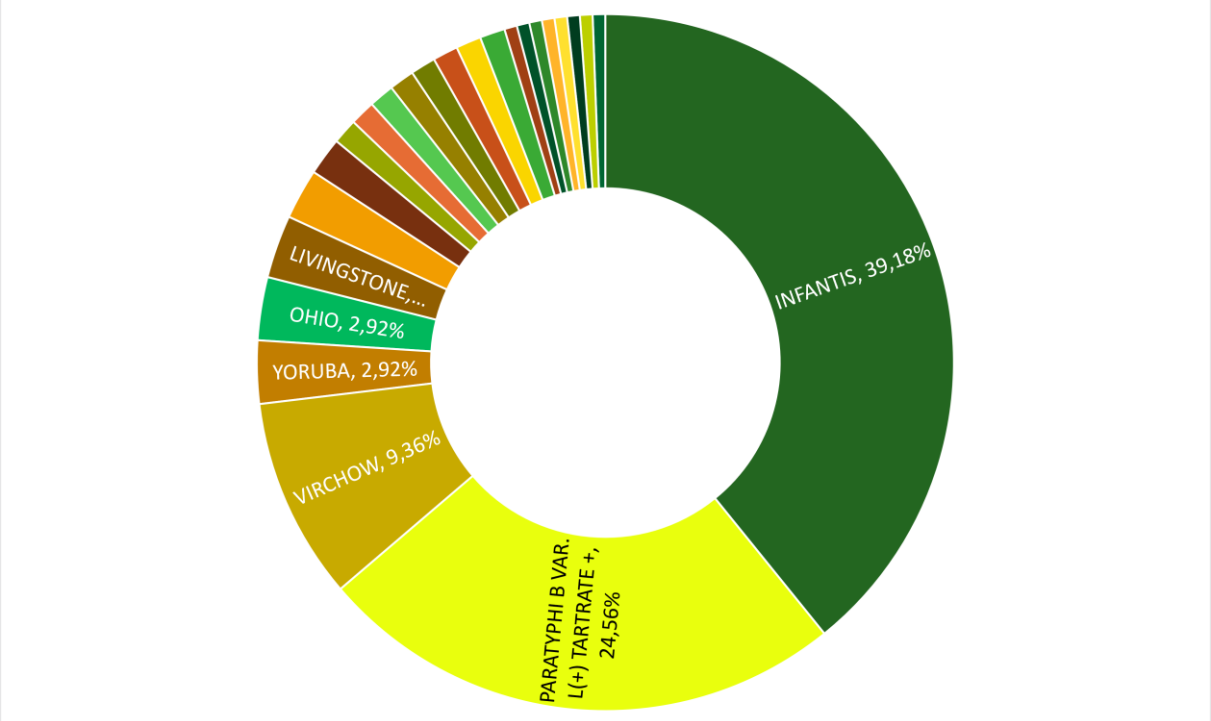


Figure 26. Percentage of serotypes identified for *Salmonella* isolated from broilers environmental samples.

Since resistance of *Salmonella* spp. varies according to the serotypes, Figure 26, shows a representation of the 24 serotypes retrieved in 2022 with an indication of the most prevalent ones. We can see that Infantis, (39,18%), Paratyphi B Var. L(+) Tartrate + (24,56%) and Virchow (9,36%) were the most prevalent serotypes in 2022.

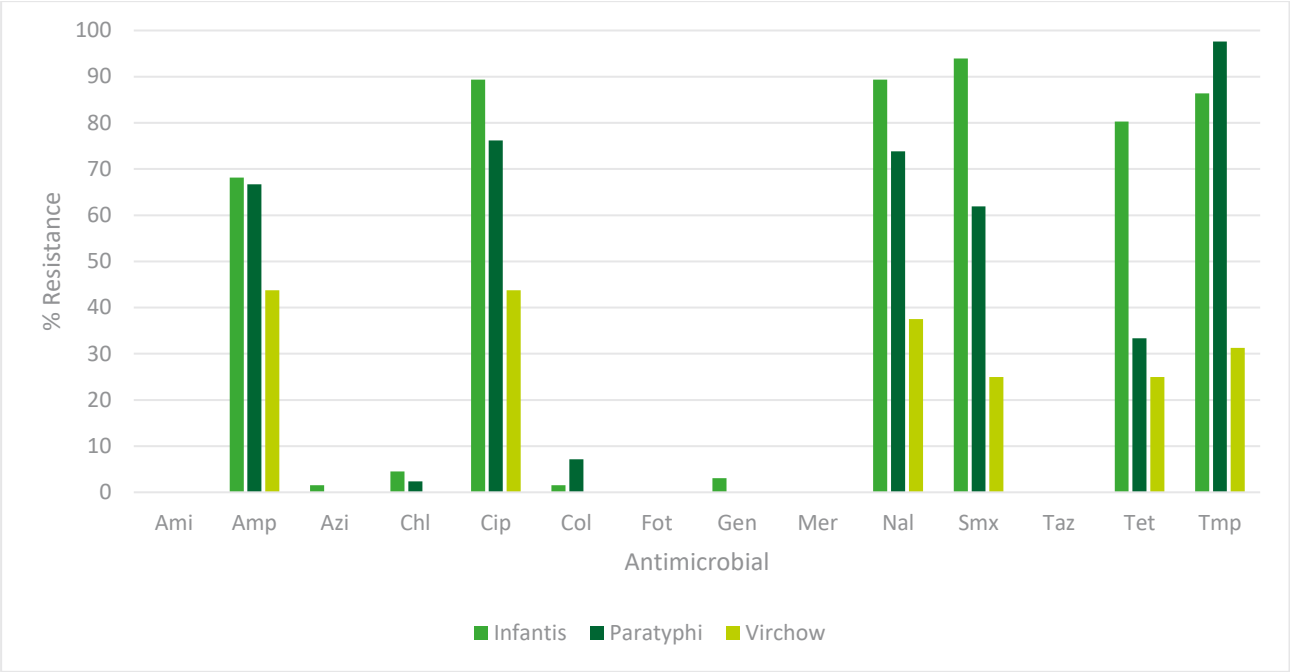


Figure 27. Comparison of resistance rates in the three most prevalent serotypes of *Salmonella* spp. retrieved from broilers environmental samples in 2022

Figure 27 shows that *S*. Infantis, while being the most prevalent serotype, also has the highest overall resistance levels. We can indeed see that resistance level to ciprofloxacin is 89,4% in *S*. Infantis. The second most resistant serotype, Paratyphi B, is also the second most prevalent and its resistance level to ciprofloxacin is 76,2% while in S. Virchowt his resistance is found in 43,8% of isolates.
This observation also applies for the resistance levels to ampicillin, chloramphenicol, nalidixic acid, sulfamethoxazole and tetracycline. We can also see that *S*. Infantis was the only serovar that had resistance to azithromycine (1,5%) and gentamicin (3%). Resistances to colistin and trimethoprim however were higher in *S*. Paratyphi B Var. L(+) Tartrate +.

Out of 659 environmental samples of laying hens tested, 30 were positive for *Salmonella* spp. and 16 were tested for antimicrobial susceptibility.

Figure 28 shows the results of AST and Figure 29 shows the prevalence of the serotypes retrieved.

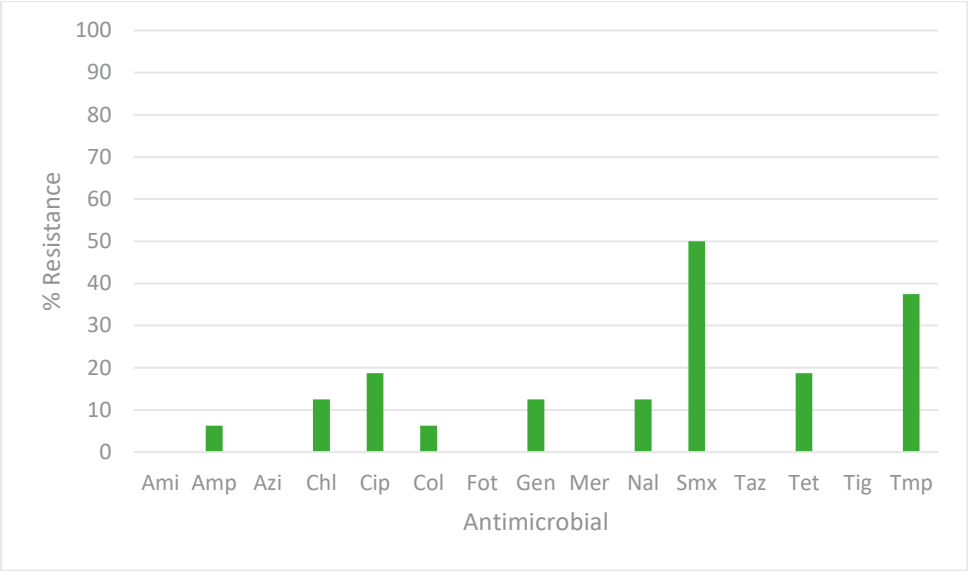


Figure 28. Comparison of resistance rates in the three most prevalent serotypes of *Salmonella* spp. retrieved from broilers environmental samples in 2022

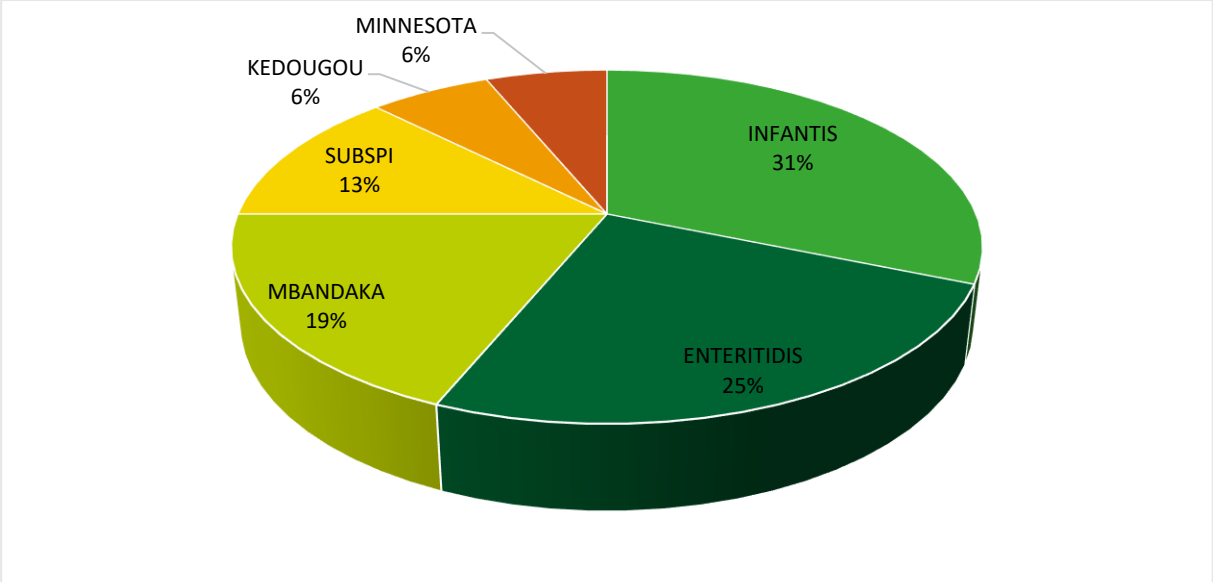


Figure 29. Percentage of serotypes identified for *Salmonella* isolated from laying hens environmental samples.

The most prevalent serotype of *Salmonella* spp. isolated from laying hens environmental samples is Infantis (31%) followed by Enteridis (25%) and Mbandaka (19%). We can see that the resistance rates are lower in laying hens than in broilers with less than 20% of isolates resistant to fluoroquinolones. Also, no resistance to azithromycin or tigecycline was detected in laying hens as opposed to broilers. Still, 6,25% of isolates were resistant to colistin. As it was the case in broilers, no resistance to meropenem, amikacin or 3rd generation cephalosporins were detected in laying hens environmental samples in 2022.

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

Table 13 shows the number of commensal indicator *E. coli* as well as ESBL/AmpC/carbapenemase producing *E.coli* isolated from food-producing animals and tested for antimicrobial susceptibility. The results of the susceptibility testing are shown below.

Table 13. Total number of commensal and ESBL/AmpC/carbapenemase *E.coli* isolated from food-producing animals and subjected to one or both panels of antimicrobials in 2022.

Programme	Technical sheet	Reported AST
Broilers - caeca	PRI 019 (broilers)	
Commensal *E. coli*		
MIC 1st panel		168
MIC 2nd panel		6
ESBL/AmpC/Carba *E. coli*		
MIC 1st panel		182
MIC 2nd panel		182
Turkeys - caeca	PRI 019 (turkeys)	
Commensal *E. coli*		
MIC 1st panel		86
MIC 2nd panel		4
ESBL/AmpC/Carba *E. coli*		
MIC 1st panel		30
MIC 2nd panel		30
Breeding hens - faeces	PRI 515	
Commensal *E. coli*		
MIC 1st panel		163
MIC 2nd panel		1
Laying hens - faeces	PRI 515	
Commensal *E. coli*		
MIC 1st panel		166
MIC 2nd panel		1
Bovines (slaughtherhouse) - caeca	PRI 036	
Commensal *E. coli*		
MIC 1st panel		166
MIC 2nd panel		4
ESBL/AmpC/Carba *E. coli*		
MIC 1st panel		180
MIC 2nd panel		180
Bovines (farm) - faeces	PRI 515	
Commensal *E. coli*		
MIC 1st panel		170
MIC 2nd panel		4
Fattening pigs - caeca	PRI 035	

Commensal *E. coli*	
MIC 1st panel	168
MIC 2nd panel	5
ESBL/AmpC/Carba *E. coli*	
MIC 1st panel	104
MIC 2nd panel	104

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

In 2022, samples of the caecal content of broilers and turkeys (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 years 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 14. Samples tested for the detection of commensal *E.coli* in 2022

Technical sheet	Description	Sampling Location	Samples Tested	*Samples positive for E. coli*
PRI 019	Broilers	Slaughterhouse	181	181
PRI 019	Turkeys	Slaughterhouse	90	90
PRI 035	Fattening pigs	Slaughterhouse	180	180
PRI 036	Bovines	Slaughterhouse	180	179
PRI 515	Bovines	Farm	177	177
PRI 515	Breeding hens	Farm	171	167
PRI 515	Laying hens	Farm	173	171

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

In 2022, 181 samples of broiler caeca were tested for the detection of *E.coli* and all were positive. Of those, 168 *E.coli* isolates were tested for antimicrobial susceptibility. The results of the minimal inhibitory concentration analysis are represented in Figure 30.

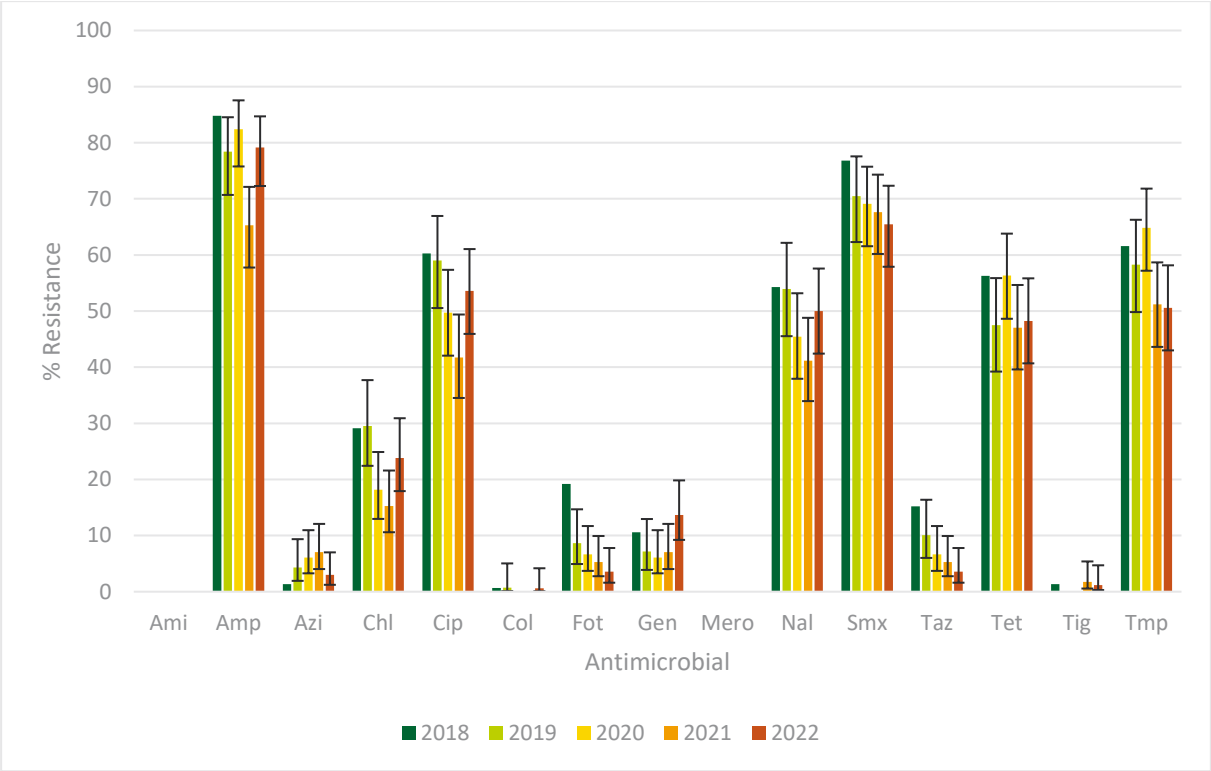


Figure 30. Trends in resistance rates to the first panel of antimicrobials in indicator *E. coli* isolated from broilers caeca (2018-2022).

In 2022, the only significant difference in comparison with 2021 is the increased resistance to ampicillin. 79% of the isolates were resistant to ampicillin, followed by sulfamethoxazole (65,48%), ciprofloxacin (53,57%), nalidixic acid and tetracycline (48%). Nevertheless, we can note decreasing trends of resistance to 3rd generation cephalosporins, sulfamethoxazole and trimethoprim. However we also see the end of the decreasing trends of resistance to (fluoro)quinolones with an increase in resistance to these antibiotics as well as to chloramphenicol and gentamicin in 2022. Ressitance to colistin was also detected in one isolate for the first time since 2019. 68% of the isolates were multidrug resistant and 7% were susceptible to all antimicrobials tested. No resistance to amikacin or meropenem was detected. 6 isolates (3,57%) showed resistance to 3rd generation cephalosporins and were therefore subjected to the analysis of the second panel of antimicrobials (EUVSEC2). The results confirmed the ESBL production : 5 of the isolates had an ESBL phenotype and the last one had a combined ESBL + AmpC phenotype.

3.2.8.2. Monitoring of indicator commensal *E.coli* in turkeys caecal content.

In 2022, 90 samples of turkeys caeca were tested for the detection of *E.coli* and all were positive. Of those, 86 *E.coli* isolates were tested for antimicrobial susceptibility. The results of the minimal inhibitory concentration analysis are represented in Figure 31.

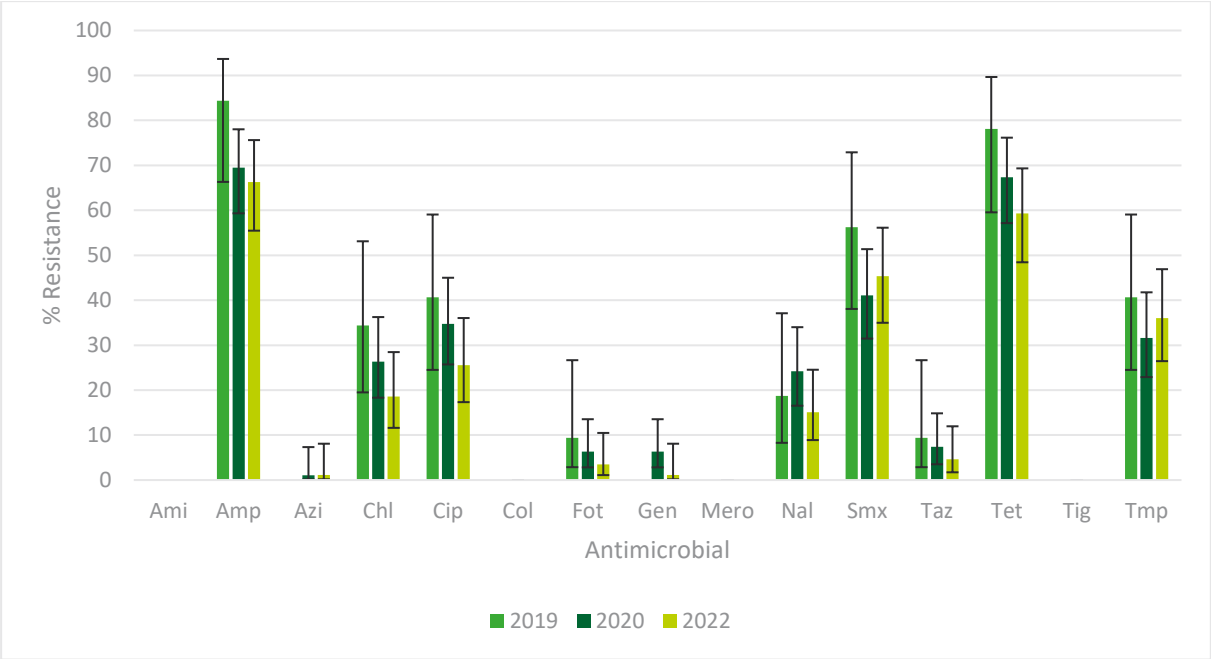


Figure 31. Trends in resistance rates to the first panel of antimicrobials in indicator *E. coli* isolated from turkeys caeca (2019-2022).

This monitoring was not conducted in 2021, so we can compare the data from 2022 to that from 2020. No significant difference was detected in this 2 years interval nor between 2019 and 2022. However, there seems to be several decreasing trends in resistance. Indeed, resistances to the critically important antimicrobials, ciprofloxacin and 3rd generation cephalosporins together with chloramphenicol and tetracycline are decreasing since 2019. 66,3% of the isolates were resistant to ampicillin, followed by tetracycline (59,3%), sulfamethoxazole (45,35%), trimethoprim (36,05%) and ciprofloxacin (25,6%). 48% of the isolates were multidrug resistant and 21% were susceptible to all antimicrobials tested. In 2022, 4 isolates showed resistance to 3rd generation cephalosporins and were tested for their suscpetibility to the antibiotics of the second panel of antimicrobials. They were confirmed as ESBL producers with 3 ESBL phenotypes and one other phenotype (resistant to ceftazidime only).

3.2.8.3. 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 2022 and all were positive. 168 of those *E.coli* isolates were tested for antimicrobial suscpetibility and the results are shown in Figure 32.

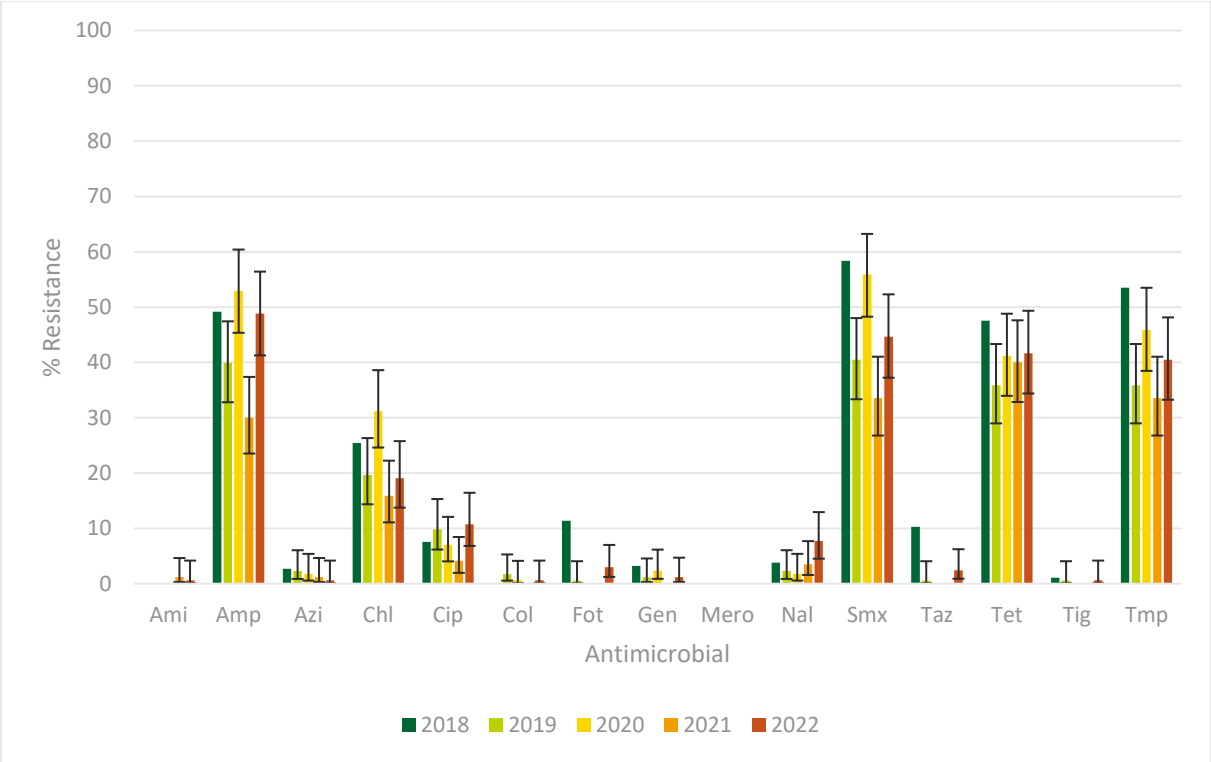


Figure 32. Trends in resistance rates to the first panel of antimicrobials in indicator *E. coli* isolated from fattening pigs caeca (2018-2022).

In comparison with 2021, we see a significant increase in resistance to ampicillin in 2022. We can also note that 5 isolates were resistant to 3rd generation cephalosporins whereas no isolate was resistant to these antibiotics in 2021 nor in 2020. Resistance to (fluoro)quinolones was also higher in 2022 with resistance to ciprofloxacin >10% for the first time in this matrix. 48,81% of the isolates were resistant to ampicillin, followed by 44,64% to sulfamethoxazole, 41,67% to tetracycline and 40,48% to trimethoprim. 41% of the isolates were multidrug resistant and 32% were susceptible to all antimicrobials tested. The 5 cephalosporin-resistant isolates were subjected to the second panel of antimicrobials (EUVSEC 2) 4 had an ESBL phenotype and the last one had a combined phenotype ESBL + AmpC.

3.2.8.4. 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 detecion of *E. coli*. Of those, 179 were positive and 166 were analysed by antimicrobial susceptibility testing. The results are shown in Figure 33.

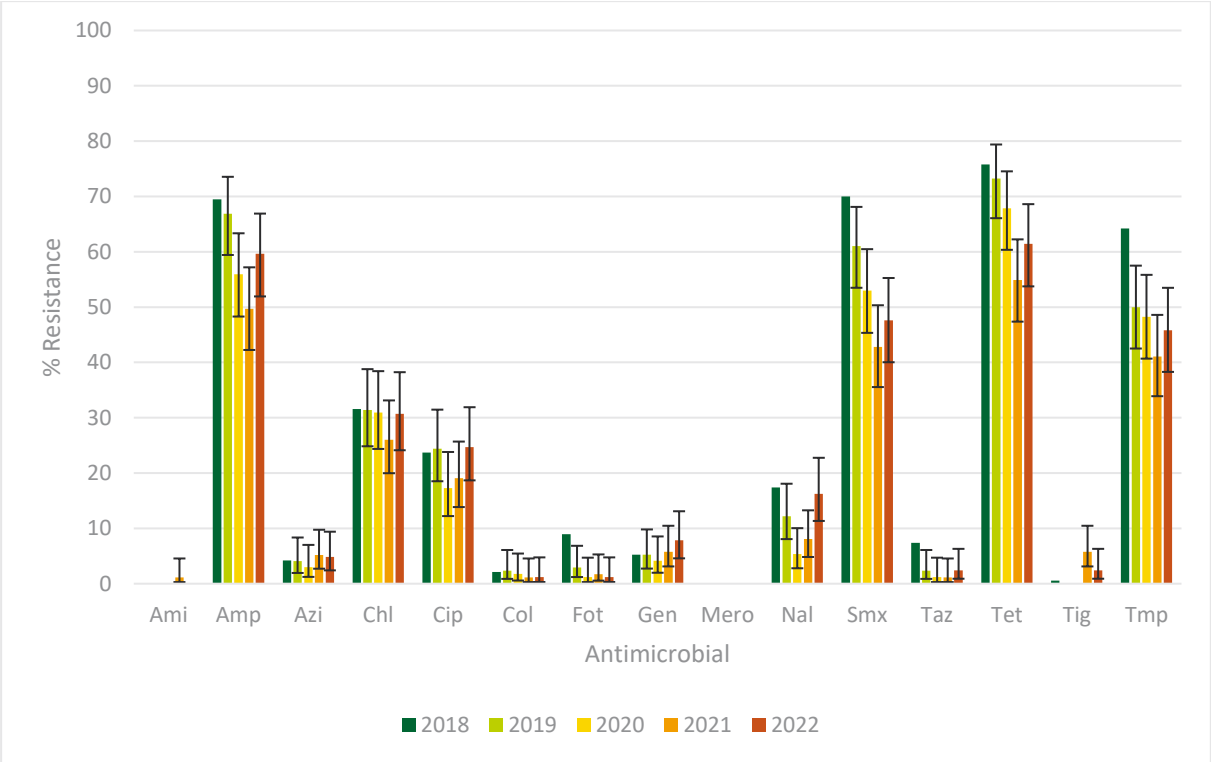


Figure 33. Trends in resistance rates to the first panel of antimicrobials in indicator *E. coli* isolated from bovines caeca (2018-2022).

Although no significant difference is seen in the results of 2022 in comparison with those of 2021, there seems to be an increase in resistances to fluoroquinolones, ampicillin, gentamicin, sulfamethoxazole, tetracycline and trimethoprim. 61,45 % of the isolates were resistant to tetracycline, followed by ampicillin (59,64%), sulfamethoxazole (47,59%) and trimethoprim (45,78%). However as opposed to 2021, no resistance to amikacin was found in 2022. 54% of isolates were multidrug resistant and 25% were fully susceptible. 4 isolates were subjected to the second panel of antimicrobials (EUVSEC2) due to their resistance to 3rd generation cephalosporins. Two isolates had an ESBL phenotype, one had an AmpC phenotype and the other one had an “other phenotype” (resistant to ceftazidime and temocillin).

3.2.8.5. Monitoring of commenal indicator *E. coli* in faeces of bovine animals less than one year old (farm)

In 2022, 177 samples of faeces from bovine animals less than one year old were collected at farm level for the detection of indicator *E.coli* and all of them were positive. Of those, 170 samples were tested for antimicrobial susceptibility. The results are shown in Figure 34.

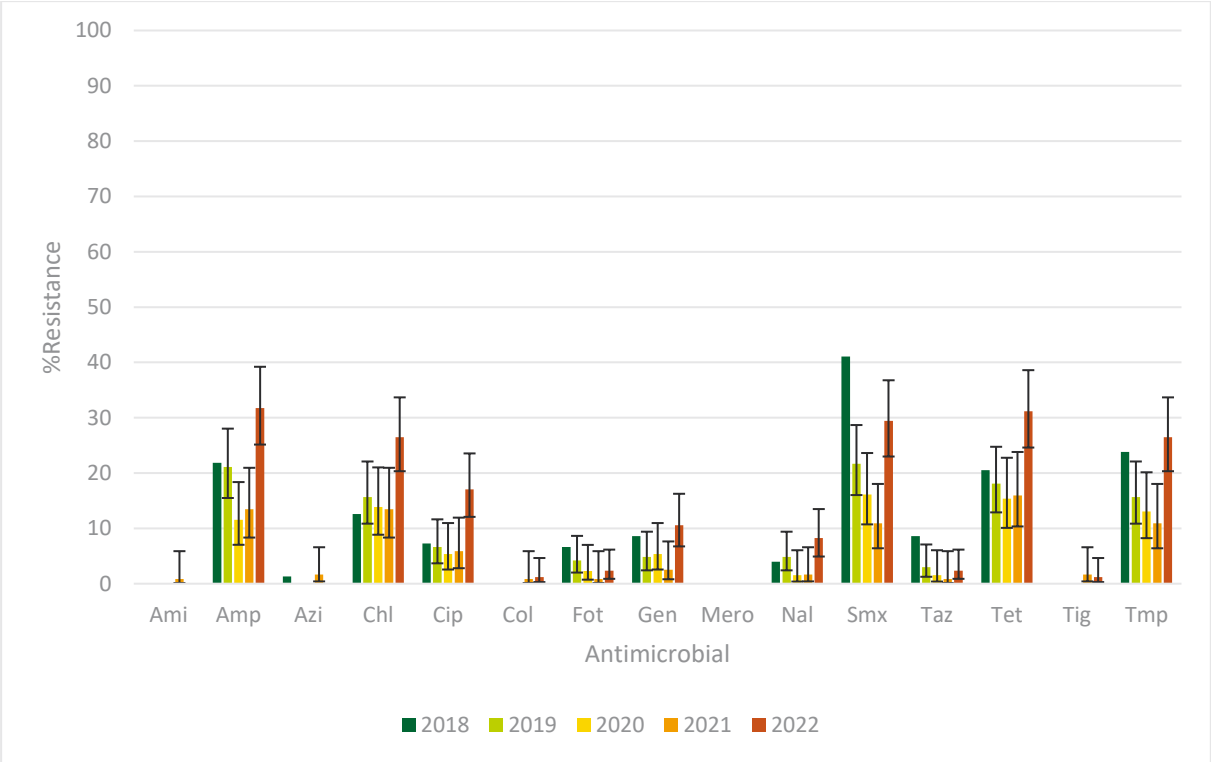


Figure 34. Trends in resistance rates to the first panel of antimicrobials in indicator E. coli isolated from bovines faeces (2018-2022).

In 2022 we see a significant increase in resistance to several antibiotics : ampicillin, ciprofloxacin, sulfamethoxazole, tetracycline and trimethoprim. 31,76% of the isolates were resistant to ampicillin, followed by tetracycline (31,18%), sulfamethoxazole (29,41%) and trimethoprim and chloramphenicol (26,47%). Regarding the critically important antimicrobial, ciprofloxacin, a significant increase is observed in 2022. However, no resistance to amikacin, azithromycin or meropenem was detected in 2022. 30% of all isolates were multidrug resistant and 54% were fully susceptible. Four isolates (2,35%) were resistant to 3rd generation cephalosporins. The testing of the second panel of antimicrobials (EUVSEC2) for these four isolates showed that two of them had an ESBL phenotype, one had an AmpC phenotype and the last one had a combination of both ESBL + AmpC phenotypes.

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

In 2022, 171 fecal samples of breeding hens were collected at farm level for the detection of *E. coli*. 167 were positive and 163 *E. coli* isolates were analysed with antimicrobial susceptibility testing. The results are shown in Figure 35.

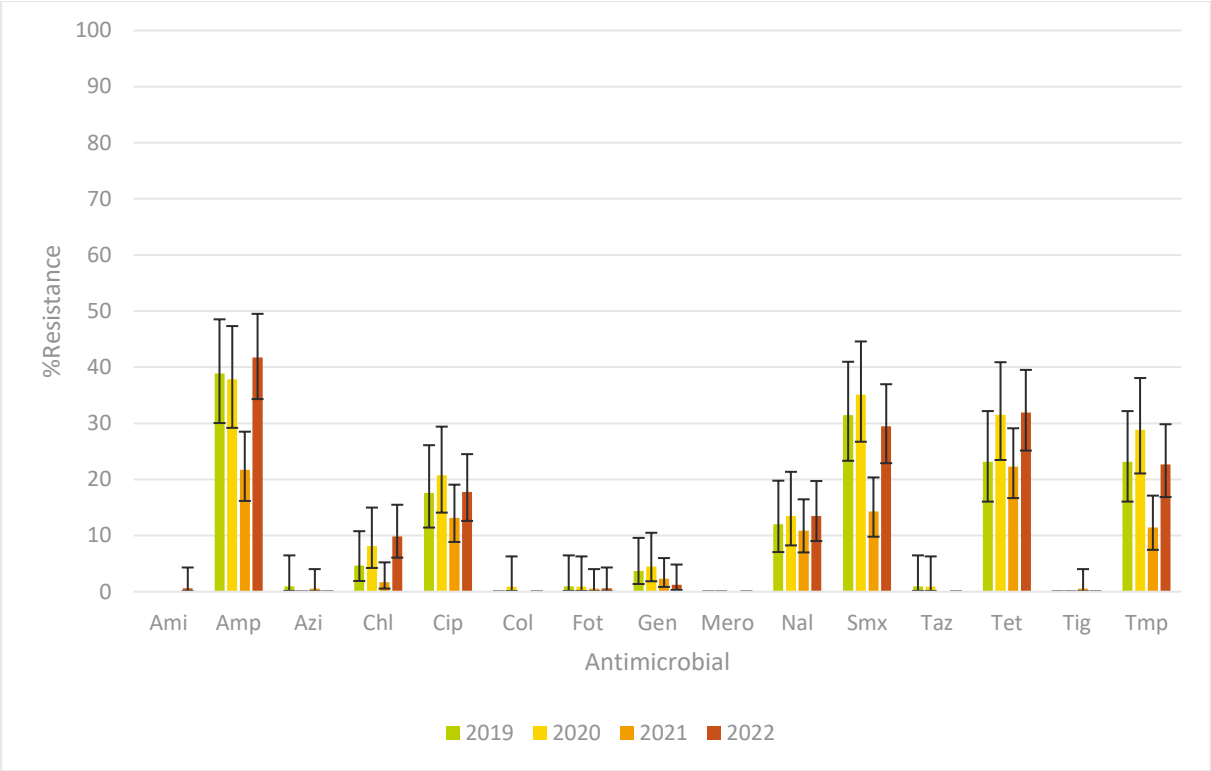


Figure 35. Trends in resistance rates to the first panel of antimicrobials in indicator *E. coli* isolated from faeces of breeding hens (2019-2022).

In 2022 many resistance rates that had decreased in 2021 increased again to levels similars as those of 2020. We can therefore note a significant increase in resistance to ampicillin, chloramphenicol and sulfamethoxazole. 41,72% of the isolates were resistant to ampicillin, followed by tetracycline (31,90%), sulfamethoxazole (29,45%) and trimethoprim (22,70%). Regarding the critically important antimicrobial, ciprofloxacin, an increase is observed in 2022. Resistance to amikacin was also detected in one isolate for the first time in this matrix in 2022. However as opposed to 2021, no isolate was resistant to tigecycline or azithromycin in 2022. Resistances to meropenem, colistin or ceftazidim were not detected either in 2022. One isolate was resistant to cefotaxime and was therefore submitted to the testing of the second panel of antimicrobials which confirmed an ESBL phenotype.

173 fecal samples of laying hens collected at farm level were also tested in 2022 for the detection of *E. coli.* 171 were positive and 166 were tested for antimicrobial susceptibility. Results are shown in Figure 36.

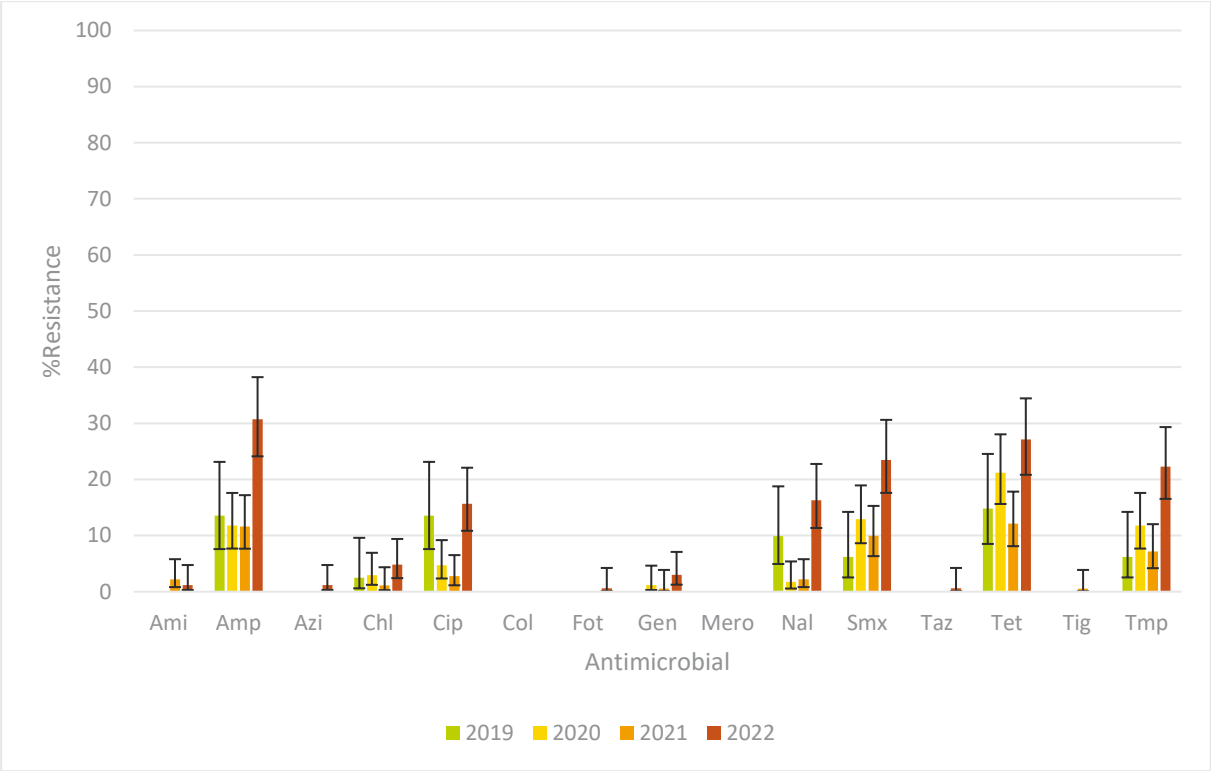


Figure 36. Trends in resistance rates to the first panel of antimicrobials in indicator *E. coli* isolated from faeces of laying hens (2019-2022).

In 2022, a significant increase in resistance to several antimicrobials was detected. Indeed, resistances to ampicillin, fluoroquinolones, tetracycline, sulfamethoxazole and trimethoprim strongly increased in 2022. 30,72% of the isolates were resistant to ampicillin, followed by tetracycline (27,11%), sulfamethoxazole (23,49%) and trimethoprim (22,29%). Resistance to ciprofloxacin has increased significantly up to 15,66%. Resistance to 3rd generation cephalosporins was also detected in one isolate for the first time in this matrix. The results of the second panel of antimicrobials confirmed an ESBL phenotype. 2 isolates (1,2%) were also resistant to azithromycin in 2022. However, no resistance to meropenem, colistin or tigecycline was detected in 2022.

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

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

In 2022, 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.

248 samples of broilers caecal content were collected at the slaughterhouse and 186 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 to 75% in 2022. No carbapenemases producing *E.coli* was detected. Antimicrobial susceptibility testing was done on 182 isolates and the results are shown in Figure 37.

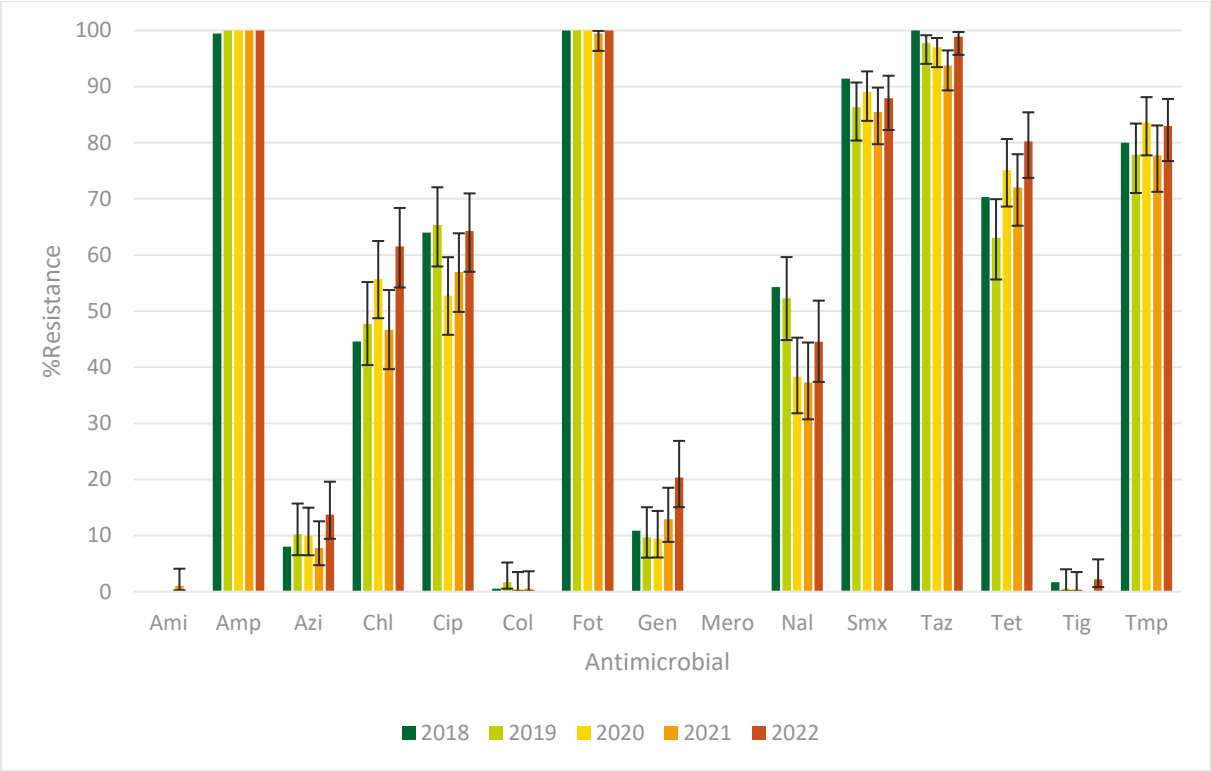


Figure 37. Trends in resistance rates to the first panel of antimicrobials in ESBL *E.coli* isolated from broilers caecal content (2018-2022).

After a decreased resistance to several antimicrobials in 2021, the resistance rates in 2022 are higher. We can note the significant increase in resistance to chloramphenicol. Resistance to critically important antimicrobial such as ciprofloxacin also seems to be an increasing trend since 2020 and resistance to nalidixic acid increased as well. Four tigecycline resistant isolates (2,2%) were found in 2022 after none had been detected in 2021. The low level of resistance observed against tigecycline may be associated with the limitation of the method, since the MIC values observed are only one dilution over the cut-off value. As opposed to 2021, no resistance to amikacin or colistin was detected in 2022. None of the isolates showed resistance to meropenem either. A worrisome feature is the very high level of resistance found in the ESBL isolates in combination with ciprofloxacin. As it is expected for ESBL producing *E. coli* the number of multidrug resistant isolates is extremely high (96,70%).

The testing of the second panel of antimicrobials allowed the characterisation of 3rd generation cephalosporins-resistant isolates according to the EFSA classification of phenotypes Figure 38).

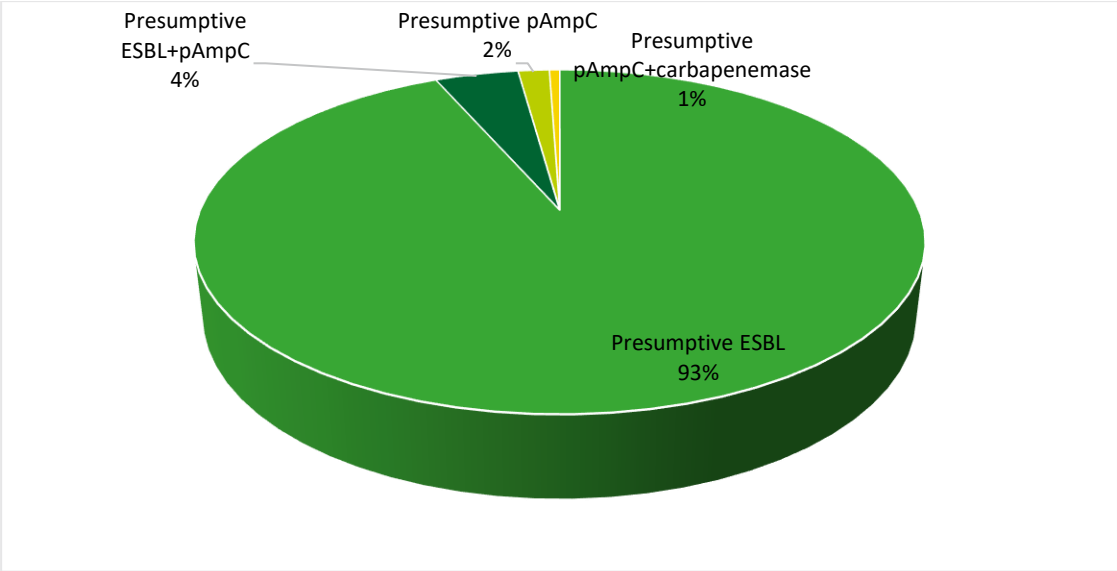


Figure 38. Percentage of phenotypes obtained using the second panel of antimicrobials in ESBL *E. coli* isolated from broilers caecal content in 2022

Most isolates (93%) had an ESBL phenotype, 2% of isolates had an AmpC phenotype and 4% of them had a combined ESBL+AmpC phenotype. One isolate was resistant to ertapenem and is therefore labelled as AmpC+carbapenemase producing *E. coli*. However since no resistance to meropenem was detected in the first or second panel, EFSA criteria don't classify it as a CP phenotype.

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

In 2022, 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.

312 samples of caecal content of fattening pigs were collected at the slaughterhouse for the detection of ESBL/AmpC producing *E.* coli. Of those, 109 were positive (34,94% compared to 40,33% in 2021) and 104 were tested for antimicrobial susceptibility (Figure 39). No carbapenemases producing *E.coli* was detected.

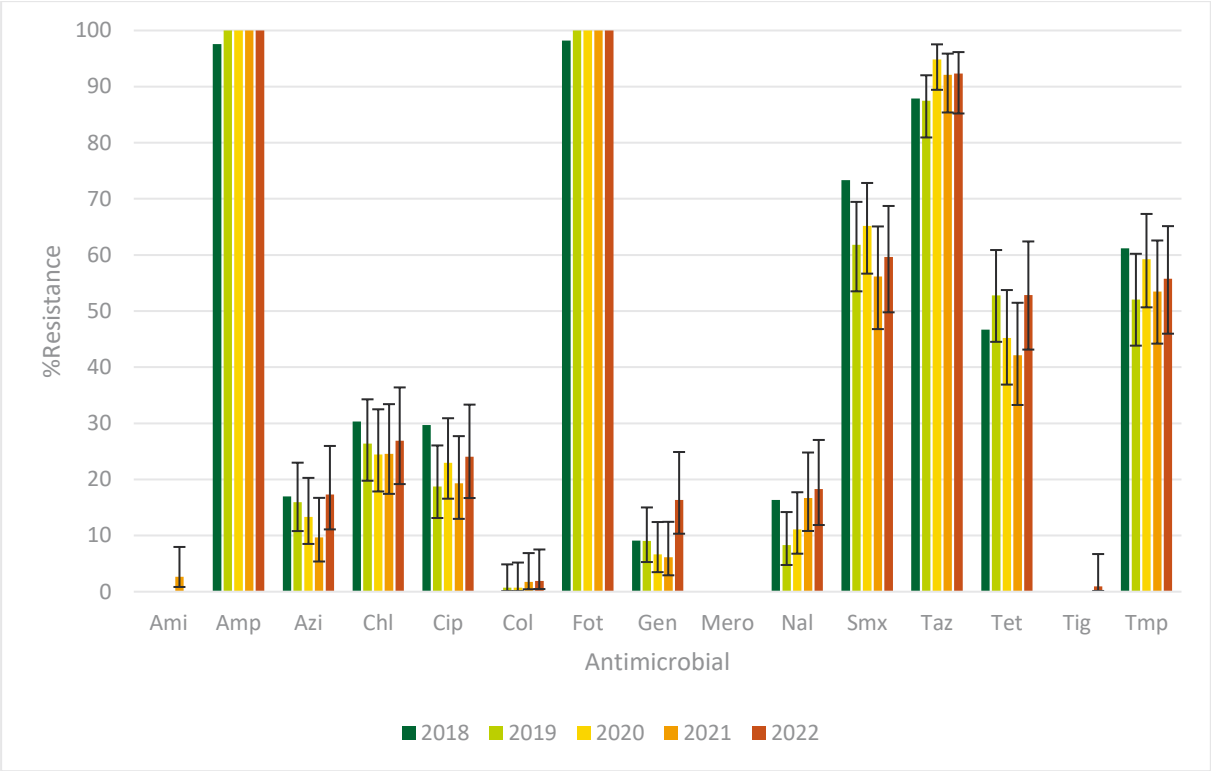


Figure 39. Trends in resistance rates to the first panel of antimicrobials in ESBL *E.coli* isolated from fattening pigs caecal content (2018-2022).

Although no significant change in resistance rates was detected in 2022, it is worth noting the detection of tigecycline resistance in one isolate for the first time in this category of food-producing animals. Most of the resistances seem to have increased in 2022. Regarding the critically important antimicrobials, resistance to ciprofloxacin has increased from 19% to 24%, similarly resistance to azithromycin has increased from 9,6% to 17,3% and gentamicin from 6% to 16,3%. However, as opposed to 2021, no resistance to amikacin was detected. No meropenem resistant isolate was detected either. 80,77% of isolates were multidrug resistant.

The characterization of the ESBL phenotype throught the analysis of the second panel of antimicrobials was conducted and the results are show in Figure 40.

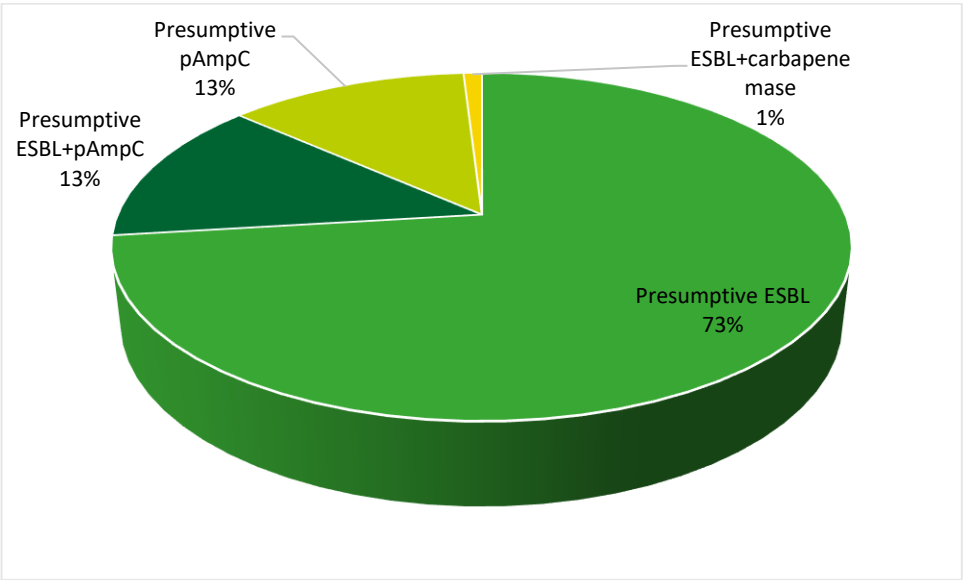


Figure 40. Percentage of phenotypes obtained using the second panel of antimicrobials in ESBL E. coli isolated from fattening pigs caecal content in 2022

73% of all isolates had an ESBL phenotype, 13% had an AmpC phenotype and 13% had a combination of both ESBL+AmpC. One isolate was resistant to ertapenem and is therefore labelled as ESBL+carbapenemase producing *E. coli*. However since no resistance to meropenem was detected in the first or second panel, EFSA criteria don't classify it as a CP phenotype.

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

In 2022, 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.

313 samples of bovine animals caeca were collected at the slaughterhouse for the detection of ESBL/AmpC producing *E. coli*. 199 samples were positive (63,58% compared to 59,80% in 2021) and 180 isolates have been subjected to antimicrobial susceptibility testing. The results are shown in Figure 41.

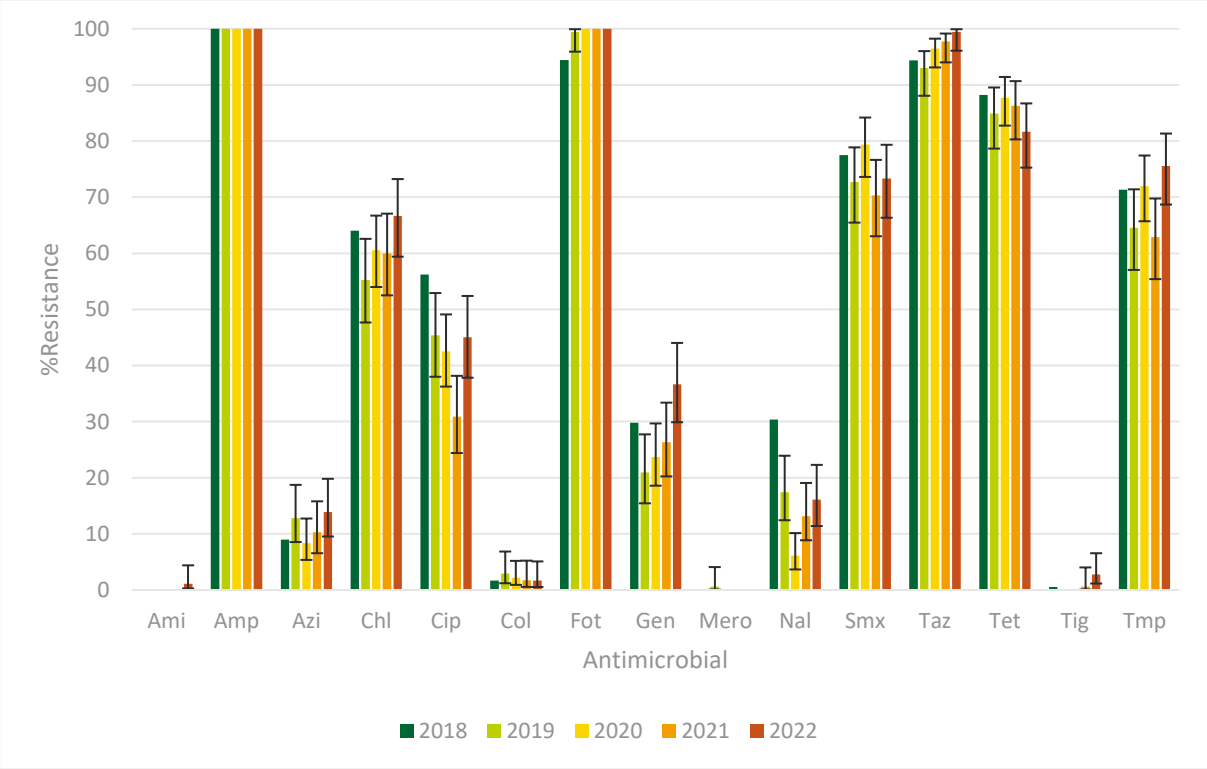


Figure 41. Trends in resistance rates to the first panel of antimicrobials in ESBL *E.coli* isolated from bovine animals caecal content (2018-2022).

No significant difference was noted in resistance rates in 2022. However, resistance to ciprofloxacin increased again in 2022 after a decreasing trend had been noticed since 2018. It is also worth mentioning the increasing trend in resistance to gentamicin since 2019. Regarding critically important antimicrobials, resistance to ciprofloxacin has increased from 30% to 45%, similarly resistance to gentamicin has increased from 26.3% to 36.67%. Two isolates (1,11%) were also resistant to amikacin. This resistance was not detected in 2021 when this antibiotic was first added to the test panel. No meropenem resistant isolate was detected in 2022. 91,11% of the isolates were multidrug resistant.

The characterization of the ESBL phenotype throught the analysis of the second panel of antimicrobials was conducted and the results are show in Figure 42.

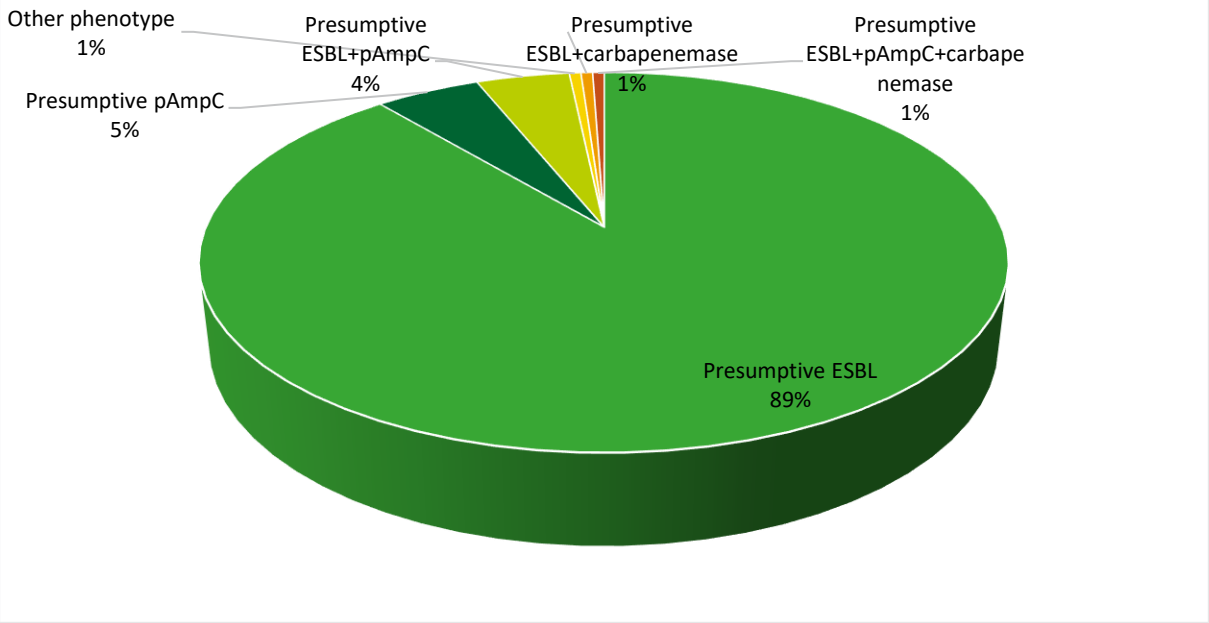


Figure 42. Percentage of phenotypes obtained using the second panel of antimicrobials in ESBL *E. coli* isolated from bovine animals caecal content in 2022

In this category of food-producing animals also, the most prevalent phenotype is ESBL (89%), 5% of isolates had an AmpC phenotype and 4% had a combined ESBL+AmpC phenotype. Moreover, 2 isolates showed resistance to ertapenem and one was thus categorised as ESBL + carbapenemase producing *E. coli* and the other one was ESBL + AmpC + carbapenemase producing *E. coli.* However since no resistance to meropenem was detected in the first or second panel, EFSA criteria don't classify these isolates as CP phenotypes. One isolate was also characterised as "Other phenotype" according to EFSA criteria, because it showed resistance to 3rd generation cephalosporins with no clavulanic acid synergy and no cefoxitin resistance.

3.2.10. Antimicrobial resistance monitoring of methicillin-resistant *Staphylococcus aureus* isolated from fattening pigs and sows

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. The latest MRSA monitoring in bovines and poultry were conducted in 2021 and 2020, respectively, and reported previously. In 2022, the monitoring focused on fattening pigs and sows to assess the MRSA prevalence in these animal categories and determine the genotypes (STs and *spa*-types) of 170 of collected MRSA isolates together with their AMR and virulence genes. Thus, the antimicrobial resistance of MRSA in 2022 was only studied genetically and no more phenotypically (no susceptibility testing).

3.2.10.1. Prevalence of methicillin-resistant *Staphylococcus aureus* in fattening pigs and sows

The yearly monitoring conducted in Belgium from 2011 to 2021 was based on the 2-S isolation method (see section 2.5). In the course of 2022 (in mid-May 2022), the isolation method used for the Belgian MRSA monitoring has been replaced by the 1-S isolation method (see section 2.5). According to the literature, the 1-S isolation method displayed in other countries a higher sensitivity for MRSA in swine samples than the "2-S" method (Larsen *et al.*, 2017). However, due to budgetary constraints, the 2 methods were not tested on the same panels of samples in Belgium and therefore the results obtained with each method could not be directly compared. Thereby, in the begin of 2022, few samples were

tested with the 2-S isolation method giving a prevalence of 23.9% (n=11/46; Ci95% [13.9-37.9%]) in sows and a prevalence of 51.3% (n=20/39, Ci95% [36.2-66.1%]) in fattening pigs. During the second part of 2022, other samples were tested with the 1-S isolation method, giving a MRSA prevalence of 52.6% (n=70/133, Ci95% [44.2-60.9%]) in sows and 87.9% (n=124/141, Ci95% [81.5-92.3%]) in fattening pigs. Based on these samples and this new 1-S method, the prevalence in fattening pigs and sows was extremely high and very high, respectively (see Table 15). In the future, the 1-S isolation method will be used and new trends analysis based on this new isolation method will be conducted.

Table 15. Prevalence of MRSA in fattening pigs and sows according to the year and the isolation method.

Animal category	Year (isolation method)	N samples	N positive	% positive	CI 95%
Fattening pigs	2022 (1-S)	141	124	87.9	81.5-92.3
	2022 (2-S)	39	20	51.3	36.2-66.1
	2019 (2-S)	180	105	58.3	51.1-65.5
	2016 (2-S)	177	112	63.3	56.2-70.4
Sows	2022 (1-S)	133	70	52.6	44.2-60.9
	2022 (2-S)	46	11	23.9	13.9-37.9
	2019 (2-S)	179	83	46.4	39.1-53.7
	2016 (2-S)	153	91	59.5	51.7-67.3

3.2.10.2. NGS investigation of methicillin-resistant *Staphylococcus aureus*

Among the 225 MRSA isolated in 2022 in Belgium, 170 isolates were analysed by whole genome sequencing. The selection of 170 out of the 225 isolates (n=72 sows, n=98 fattening pigs isolates) followed the global stratification of the sampling by local control unit and was evenly divided over the year among the sows and fattening pigs isolates, separately.

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

In sows, all but five isolates belonged to ST398 and to the following spa-types: t011 (43), t034 (14), t2011 (3), t1451 (1), t1457 (1), t15528 (1), t3423 (1), t5104 (1), t588 (1), t6575 (1). The remaining isolates belonged to ST3706/t011 (2), ST7645/t034 (1) or newly described ST8149/t011 (1), ST8151/t034 (1) sequence-types/spa-types.

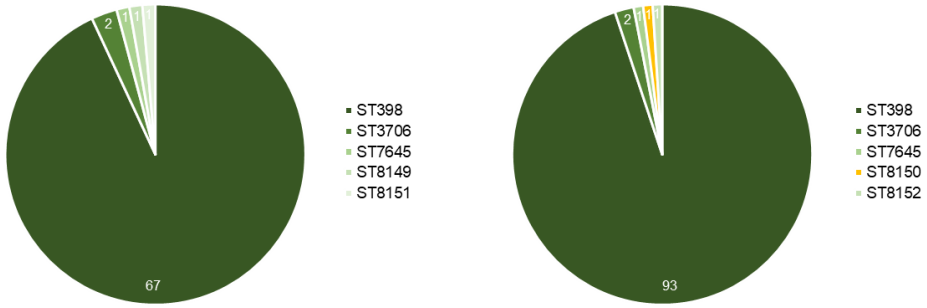
In fattening pigs, 93 isolates belonged to ST398 and to the following spa-types: t011 (60), t034(24), t1580 (2), t2011 (2), t1255 (1), t1451 (1), t3423 (1) and t779 (1). One last ST398-isolate exhibited an unknown spa-type, differing from t011 by only the first repeat (r56). The 5 remaining isolates belonged to ST3706/ t011 (2), ST7645/t034 (1) and newly described ST8150/t011 (1) and ST8152/t034 (1) sequence-types/spa-types (see Table 16).

Thus, 4 new STs were observed among the MRSA isolated in 2022: 3 of these (ST8149, ST8151 and ST8152) had only one out of the 7 loci different than ST398 (see Figure 43). Such single-locus variants are considered to belong to the same clonal complex according to Feil *et al.* (2004) and thus belong to the CC398 LA-MRSA clonal complex. The last new ST (ST8150) had 2/7 loci different than ST398. A clonal complex has not yet been assigned to this latter. However, this isolate is likely to belong to LA-MRSA given its LA-MRSA spa-type: t011. This is also supported by the genetic closeness of the ST8150 isolate to ST398 isolates, which was observed through the comparison of the ST8150 isolate with a few isolates from CC398, CC1 and CC8 based on core genome MLST typing phylogeny (see Table 17). Besides this isolate, the other 169 MRSA analyzed in 2022 are all genotyped as LA-MRSA according to their STs/spa-types combinations.

Table 16. Number of MRSA isolates and percentages of different spa-types observed in the different animal categories.

Animal category	N total		t011	t034	t588	t779	t1255	t1451	t1457	t1580	t2011	t3423	t5104	t6575	T15528	Unknown*
Fattening pigs	98	N	63	26	0	1	1	1	0	2	2	1	0	0	0	1
		%	64.3	26.5	0.0	1.0	1.0	1.0	0.0	2.0	2.0	1.0	0.0	0.0	0.0	1.0
Sows	72	N	46	16	1	0	0	1	1	0	3	1	1	1	1	0
		%	63.9	22.2	1.4	0.0	0.0	1.4	1.4	0.0	4.2	1.4	1.4	1.4	1.4	0.0
Total	170	N	109	42	1	1	1	2	1	2	5	2	1	1	1	1
		%	64.1	24.7	0.6	0.6	0.6	1.2	0.6	1.2	2.9	1.2	0.6	0.6	0.6	0.6

*One fattening pig isolate exhibited an unknown spa-type, differing from t011 by only the first repeat (r56).



Sows (n=72) **Fattening pigs (n=98)**

Figure 43. Sequence-types identified in MRSA isolated from fattening pigs and sows in 2022. The numbers indicated in the charts correspond to the number of isolates characterized by each ST.

Table 17. Number of alleles differing between MRSA isolates CC1 (VAR-708), CC8 (VAR-707), CC398 (VAR-986, VAR-1001) and MRSA likely related to CC398 (VAR-997).

	cgmlst-VAR-986	cgmlst-VAR-997	cgmlst-VAR-1001	cgmlst-VAR-707	cgmlst-VAR-708
cgmlst-VAR-986	0	21	31	1748	1761
cgmlst-VAR-997	21	0	24	1748	1761
cgmlst-VAR-1001	31	24	0	1750	1763
cgmlst-VAR-707	1748	1748	1750	0	1461
cgmlst-VAR-708	1761	1761	1763	1461	0

This table of allele distances was calculated based on the 1888 remaining loci after removing the low quality loci. The numbers indicate the number of alleles differing between the cgMLST profiles of the 5 isolates.

- **Antimicrobial resistance genes**

The 170 MRSA were harboring the *mecA* gene mediating methicillin resistance, which is typical of LA-MRSA. In addition, with the exception of seven isolates (n=2 sows and n=5 fattening pigs), all were characterized by the presence of *blaZ* conferring resistance to penicillin.

Among tetracycline resistance genes, the four *tet38, tetK, tetL* and *tetM* genes were found in 100%, 77.8%, 6.9%, 98.6% of sows isolates and 100%, 77.6%, 24.5% and 99.0% of fattening pigs isolates, respectively. At least one of the tetracycline resistance genes was detected per isolate in 2022. The two *tet38* and *tetM* genes were found together in all but two isolates (1 sow and 1 fattening pig).

Eleven different aminoglycosides resistance genes were identified in MRSA isolated from sows and fattening pigs as follows: *aac(6')-aph(2'')* (13.9% and 16.3%), *aadD* (5.6% and 19.4%), *ant(6)-Ia* (2.8% and 2.0%), ant(9)-Ia (30.6% and 38.8%), *aph(2'')-Ic* (0.0% and 1.0%), *aph(3')-IIIa* (1.4% and 0.0%), *apmA* (5.6% and 10.2%), *sat4* (1.4% and 0.0%), *spd* (6.9% and 7.1%), *spw* (2.8% and 2.0%) and *str* (1.4% and 2.0%), respectively in sows and fattening pigs. Globally, 58.3% of sows isolates and 70.4% of fattening pigs isolates were harboring at least one of the aminoglycosides resistance genes.

The two *dfrG* and *dfrK* genes, reported to confer resistance to diaminopyrimidines were detected in 29.2% and 55.6% of sows isolates and in 24.5% and 65.3% of fattening pigs isolates, respectively. These genes were not detected together in the same isolates. Thus, 84.7% of sows isolates and 89.8% of fattening pigs isolates were harboring a diaminopyrimidines resistance gene.

The phenicols resistance gene *fexA* was identified in 6.9% of sows and 8.2% of fattening pigs isolates. *fexA* was detected in all the three isolates harboring *cfr*, also reported to confer this resistance.

Five genes belonging to the *erm* genes family, coding for 23S rRNA methylases and mediating resistance to macrolides, lincosamides and streptogramins B were identified in MRSA from sows and fattening pigs as follows: *erm(54)* (0.0% and 2.0%), *erm(A)* (1.4% and 8.2%), *erm(B)* (9.7% and 21.4%), *erm(C)* (15.3% and 10.2%) and *erm(T)* (0.0% and 4.1%), respectively. Thus, at least one *erm* gene was detected in 26.4% of sows isolates and in 39.8% of fattening pigs isolates.

Streptogramins resistance genes were also detected in MRSA with *lsa(E)* found in 29.2% of sows isolates and in 28.6% of fattening pigs isolates and with *vga(C)* and *vga(E)* found in 2.0% and 6.1% of fattening pigs isolates respectively.

The three genes *lnu(A)*, *lnu(B)* and *lnu(G)*, mediating lincosamides resistance, were observed in sows (5.6%, 29.2% and 5.6%) and in fattening pigs (0.0%, 28.6% and 2.0%) isolates. The presence of *lsa(E)*, a gene also reported to confer resistance to lincosamides and pleuromutilins, was detected in all isolates harboring *lnu(B)* and not in the isolates without *lnu(B)* (co-occurrence of these 2 genes). Also, the *vga(C)* and *vga(E)* genes cited hereabove also confer resistance to lincosamides and pleuromutilins.

Three isolates (n=1 sows and n=2 fattening pigs) harbored a critically important antibiotic (linezolid) resistance gene : *cfr*, also reported to confer resistance to phenicols, lincosamides, streptogramins A and pleuromutilins, in addition to the resistance to the oxazolidinones class (a.o. linezolid).

The *bleO* gene conferring resistance to bleomycin, a glycopeptide antibiotic, was detected in one fattening pig isolate. This isolate was tested phenotypically by broth microdilution with EUST2 Sensititre plate and was found fully susceptible to vancomycin (MIC≤1 mg/L). Thus, this gene was not associated with a phenotypic resistance to vancomycin. Moreover, as far as our knowledge, the bleomycin is used in human medicine as an anti-tumour treatment rather than as an antibiotic.

In 2022, several genes mediating resistance to more than one antimicrobial class were detected. This was particularly observed for lincosamides, streptogramins and pleuromutilins classes. Considering this and per antimicrobial class, many isolates harbored at least one resistance gene as follows: 51.4% of sows and 60.2% of fattening pigs isolates with at least one streptogramins resistance gene, 61.1% of sows and 62.2% of fattening pigs isolates with at least one lincosamides resistance gene and 29.2% of sows and 37.8% of fattening pigs isolates with at least one pleuromutilins resistance gene (see Figure 44, Figure 45 and Annex Ia).

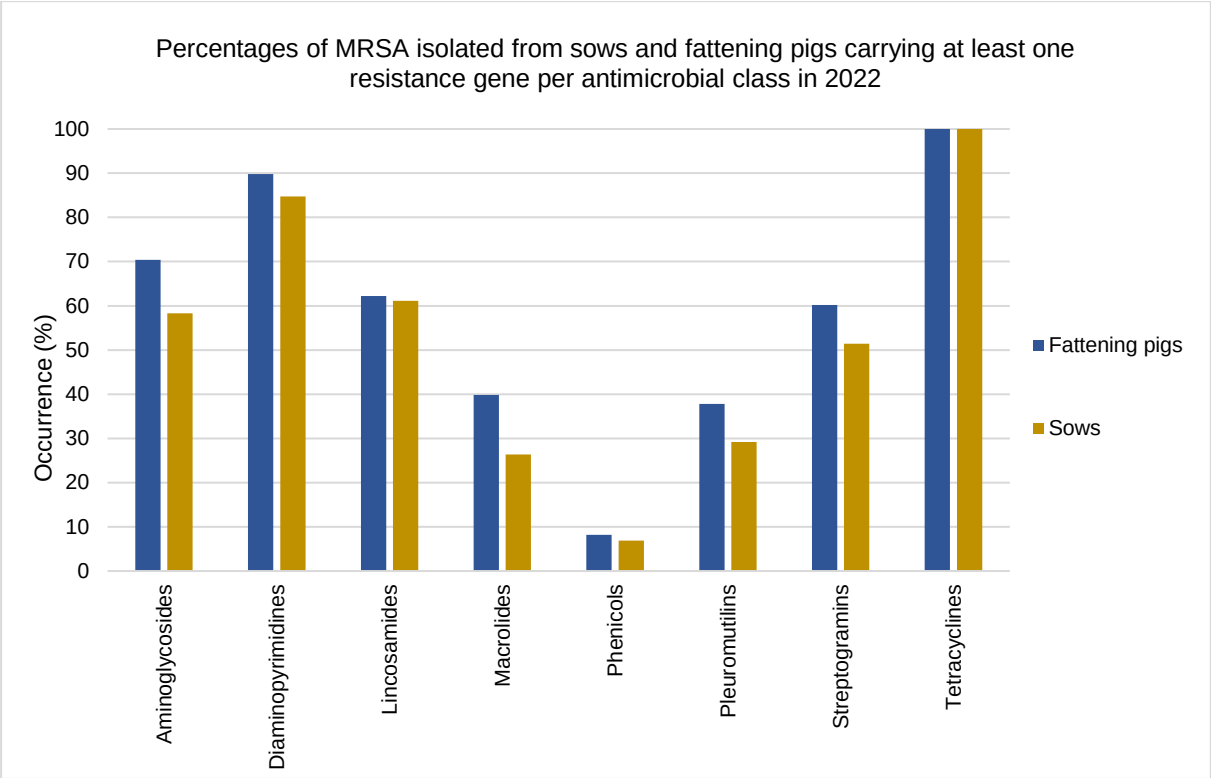


Figure 44. Percentages of MRSA isolated from fattening pigs and sows carrying at least one resistance gene per antimicrobial class in 2022.

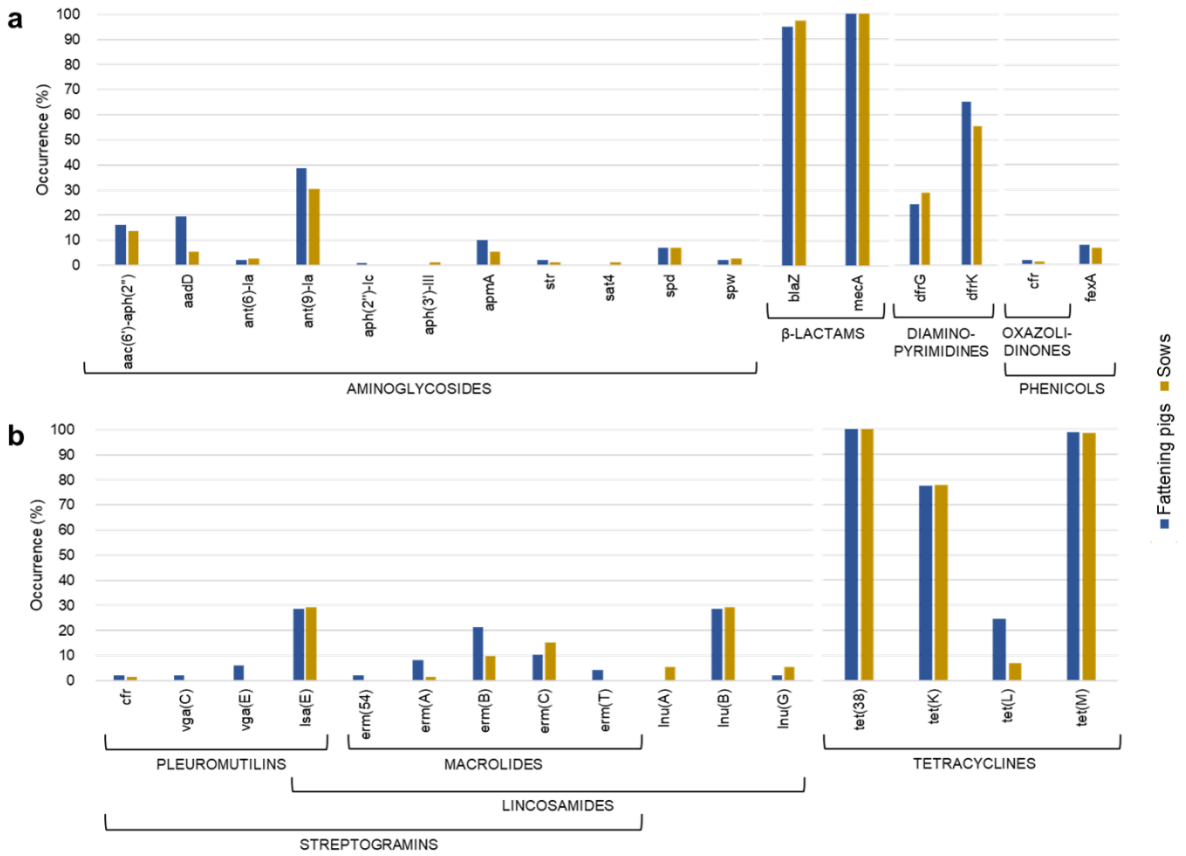


Figure 45. Occurrence of resistance genes observed in MRSA isolated from fattening pigs and sows in 2022 and classified per antimicrobial class, (a) aminoglycosides, β-lactams,

diaminopyrimidines, oxazolidinones, phenicols and (b) macrolides, lincosamides, streptogramins, pleuromutilins and tetracyclines.

- **Biocide resistance genes**

In addition to the detection of antimicrobials resistance genes, the Resfinder tool used allow also the detection of some biocide resistance genes. By this way, *qac* genes mediating resistance to quaternary ammonium compounds were observed in MRSA in 2022: *qacG* was found in 6.9% and 6.1% of sows and fattening pigs isolates respectively, and *qacJ* in 3.1% of fattening pigs. The *qacG* and *qacJ* genes were not detected in the same isolates (see Annex Ib).

- **Virulence genes**

In 2022, several virulence genes were observed among the collection of MRSA isolates from sows and fattening pigs: genes associated with toxins (*selw, seb, 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 170 isolates: *aur, selw, hlgB* and *hlgC*. The toxins associated gene *hlgA* was detected in all but one isolates (absent in one ST398/t011 sow isolate). Conversely, the *seb* gene was detected in a single isolate (ST398, t011) from fattening pigs. In addition, one sow isolate belonging to ST398/t034 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. Noteworthy, the *seb* gene found in a single isolate from fattening pigs in 2022 encodes the Staphylococcal enterotoxin B (SEB) which is a protein exotoxin found on the cell surface of *S. aureus* that is the source for multiple pathologies in humans (Verreault *et al.,* 2019). This gene has already been reported in the literature in *S. aureus* isolates from pigs, among others in Poland and in Latvia (Bystron *et al*, 2015; Ivbule, 2019).
Among the most frequently observed virulence genes observed in the 2022 MRSA monitoring: 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* were detected in all 2021 bovine isolates analysed by whole-genome sequencing and are thus common in Belgian LA-MRSA.

3.2.10.3. Discussion

In 2022, all but one isolate were genotyped as LA-MRSA according to their STs/*spa*-types combinations. A clonal complex has not yet been assigned to the latter. However, this isolate is likely to belong to LA-MRSA according to our investigation presented hereabove.
The presence of MRSA in food-producing animals and their carriage of several AMR and virulence genes represents a public health risk. The extremely high prevalence (87.9%) observed in fattening pigs in 2022 with the new isolation method, as well as the carriage of the linezolid resistance *cfr* gene observed again in 2022 in a few isolates (n=3), are therefore a matter of concern. This *cfr* gene mediating resistance to the critically important antibiotic linezolid was already reported in 5 *S. aureus* isolated in 2013 (n=1), 2016 (n=2) and 2019 (n=2) from nasal swabs from pigs and sows in Belgium (Timmermans *et al*., 2022).
Moreover, several virulence genes associated with the immune evasion cluster (*sak, scn*), associated with toxins (*hlgA, hlgB, hlgC, seb* and *selw*) and exoenzymes (*aur*) were detected among the 170 MRSA isolates collected in 2022 and characterized through whole-genome sequencing. In 2022, one sow isolate belonging to the ST398-t034 LA-MRSA type 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 carried the *tet(M)* gene, which is also typical of LA-MRSA and did not carry critically antimicrobial resistance gene (no *cfr* gene), neither *qac* disinfectant resistance gene. Altogether, this isolate would probably not have been recently transmitted from humans to sows, given the livestock-associated genetic background observed. The genes associated with the immune evasion

cluster were not observed in the other isolates. The detection of the *seb* gene encoding an exotoxin known to be the source for multiple pathologies in humans in an isolate from fattening pigs highlights the importance of monitoring these different virulence factors in the future.

Several changes in the methodology used for the monitoring of MRSA have been made in 2022, including a new isolation method and the study of AMR through NGS rather than phenotypic susceptibility testings. The 2022 data will now serve as a new baseline for analyzing future trends in the prevalence of MRSA and the AMR genes they carry.
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.

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

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

From 269 broiler samples, *E. faecalis* was isolated in 176 samples (65.4%) and *E. faecium* in 152 samples (56.5%). *E. faecalis* (92.0%, n=92) was twice more often isolated than *E. faecium* (40.0%, n=40) in turkey samples. In layer and breeder samples, *E. faecium* was more often isolated than *E. faecalis*. From 180 breeder samples, *E. faecium* was isolated in 154 samples (85.6%) and *E. faecalis* in 51 samples (28.3%). Similarly, among 226 layer samples, *E. faecium* was isolated in 158 of these samples (i.e. 69.9%) and *E. faecalis* in 111 (i.e. 49.1%). Among 253 veal calve samples, *E. faecium* was isolated in 64.0% of these samples (n=162) while *E. faecalis* was isolated in 32.8% of them (n=83). From 268 pig samples, *E. faecium* was isolated up to 3 times more often than *E. faecalis* with n=178 (66.4%) and n=51 (19.0%) respectively (see Figure 46).

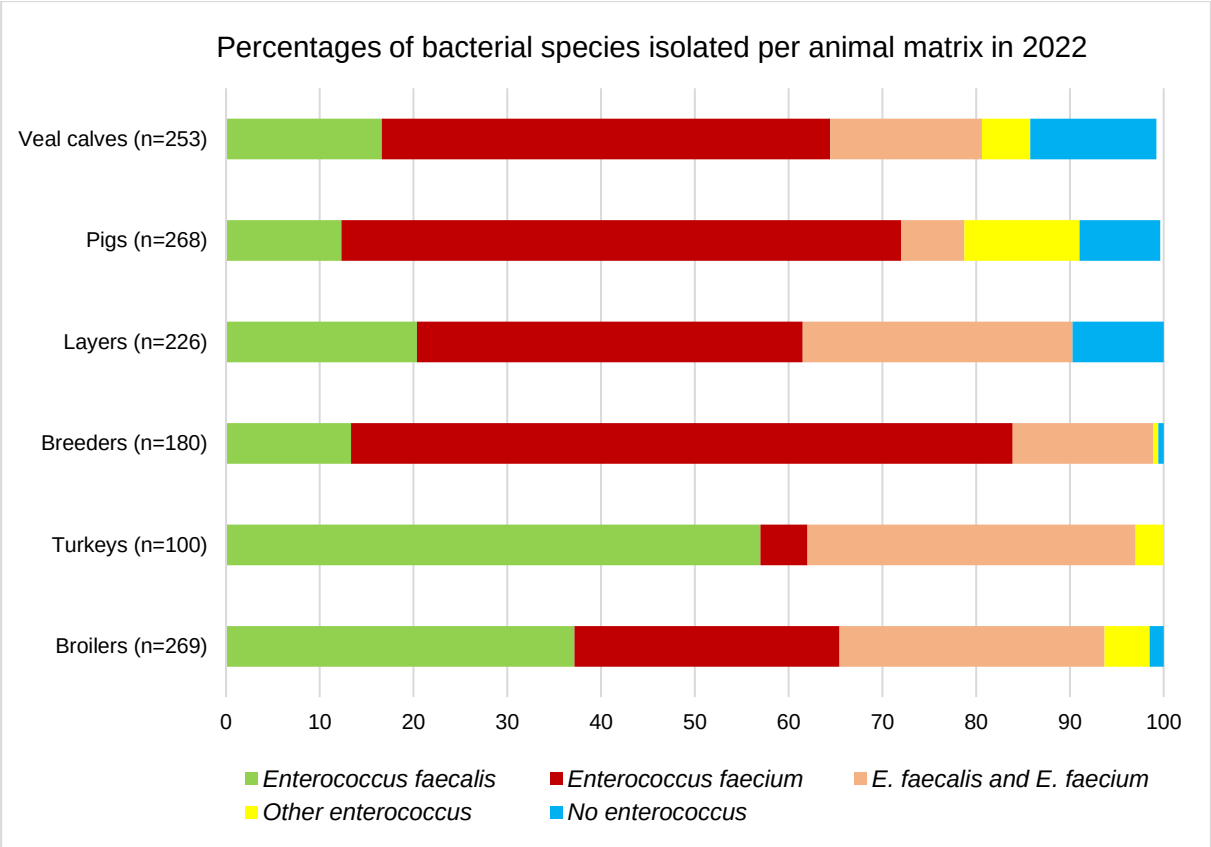


Figure 46. Prevalence of *Enterococcus faecalis* and *Enterococcus faecium* isolation per animal matrix in 2022.

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

- Broiler samples collected at the slaughterhouse

A total of 321 antimicrobial susceptibility tests were performed after isolation of *Enterococcus faecalis* (n=170) and *Enterococcus faecium* (n=151) from broiler samples.

Within these samples, very high to extremely high levels of resistance were observed for erythromycin, quinupristin/dalfopristin and tetracycline. Indeed, 90.0% of *E. faecalis* and 68.9% of *E. faecium* showed resistance to tetracycline; as well as 82.9% of *E. faecalis* and 72.2% of *E. faecium* with erythromycin resistance; and 80.1% of *E. faecium* isolates resistant to quinupristin/dalfopristin. Moreover, the occurrence of ampicillin resistance was moderate, observed in 11.3% of *E. faecium*, only. The level of resistance observed to ciprofloxacin (5.3%) was low in *E. faecium*. Similarly, observed only in *E. faecium*, resistance to gentamicin was very low (0.7%).

Following the modification of the resistance threshold (from 4 to 8 mg/L) to daptomycin applied for *E. faecium* in 2021, this resistance was absent from broilers in 2022. Resistance to daptomycin was also absent in *E. faecalis*. No resistance to chloramphenicol, linezolid, teicoplanin, tigecycline or vancomycin was observed in broilers in 2022 (see Figure 47).

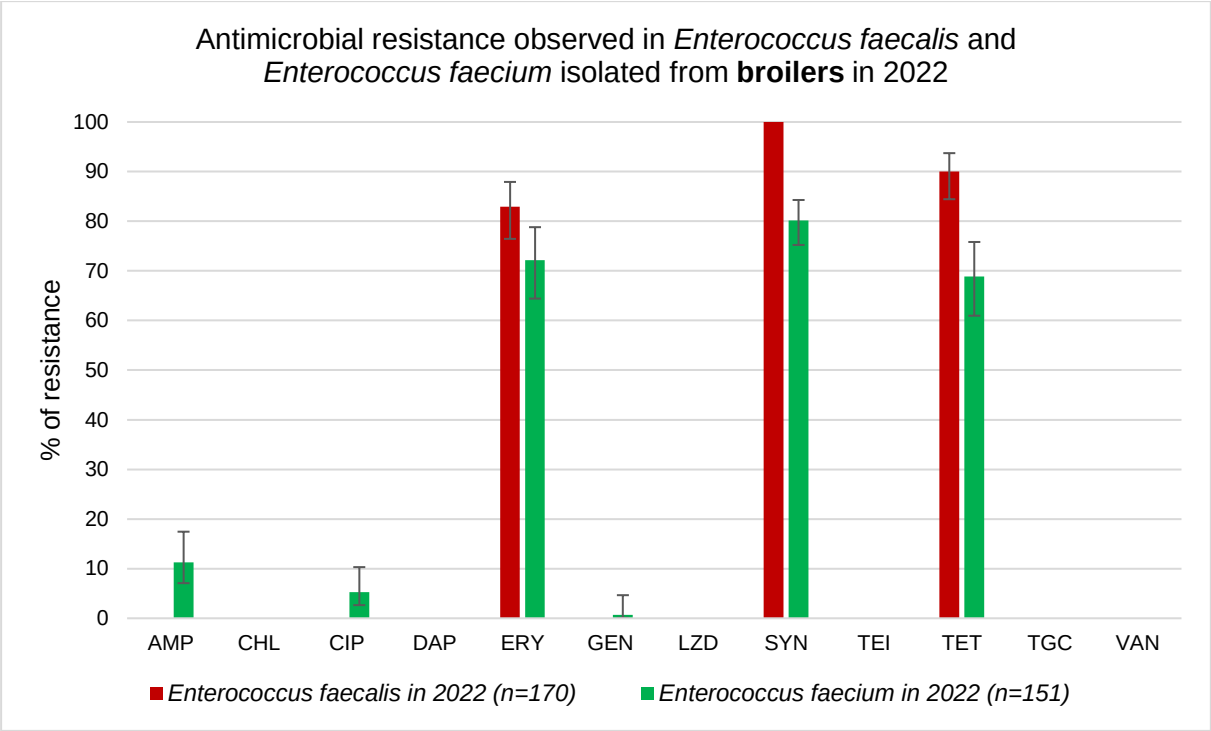


Figure 47. Percentages of antimicrobial resistance in *Enterococcus faecalis* (n=170) and *Enterococcus faecium* (n=151) isolated from broilers in 2022.

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).

- Turkey samples collected at the slaughterhouse

A total of 132 antimicrobial susceptibility tests were performed after isolation of *Enterococcus faecalis* (n=92) and *Enterococcus faecium* (n=40) from turkey samples.

Within these samples, very high to extremely high levels of resistance were observed for erythromycin, quinupristin/dalfopristin and tetracycline. Indeed, 91.3% of *E. faecalis* and 63.4% of *E. faecium* showed resistance to tetracycline; as well as 68.5% of *E. faecalis* and 56.1% of *E. faecium* with erythromycin resistance; and 78.0% of *E. faecium* resistant to quinupristin/dalfopristin. Regarding resistance to quinupristin/dalfopristin, although intrinsic in *E. faecalis*, the rate was 95.7% in *E. faecalis* isolated from turkeys, based on the resistance threshold set at > 0.5 mg/L by EFSA (https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/sp.efsa.2021.EN-6652).

In addition, the occurrence of ampicillin resistance was moderate, observed in 17.1% of *E. faecium*, only. The level of resistance observed to ciprofloxacin (9.8%) was low in *E. faecium*. On the contrary, observed only in *E. faecalis*, resistance to gentamicin was low as well (1.1%).

Following the modification of the resistance threshold (from 4 to 8 mg/L) to daptomycin in *E. faecium* in 2021, this resistance was absent among strains isolated from turkeys in 2022. No resistance to chloramphenicol, linezolid, teicoplanin, tigecycline or vancomycin was observed in turkeys in 2022 (see Figure 48).

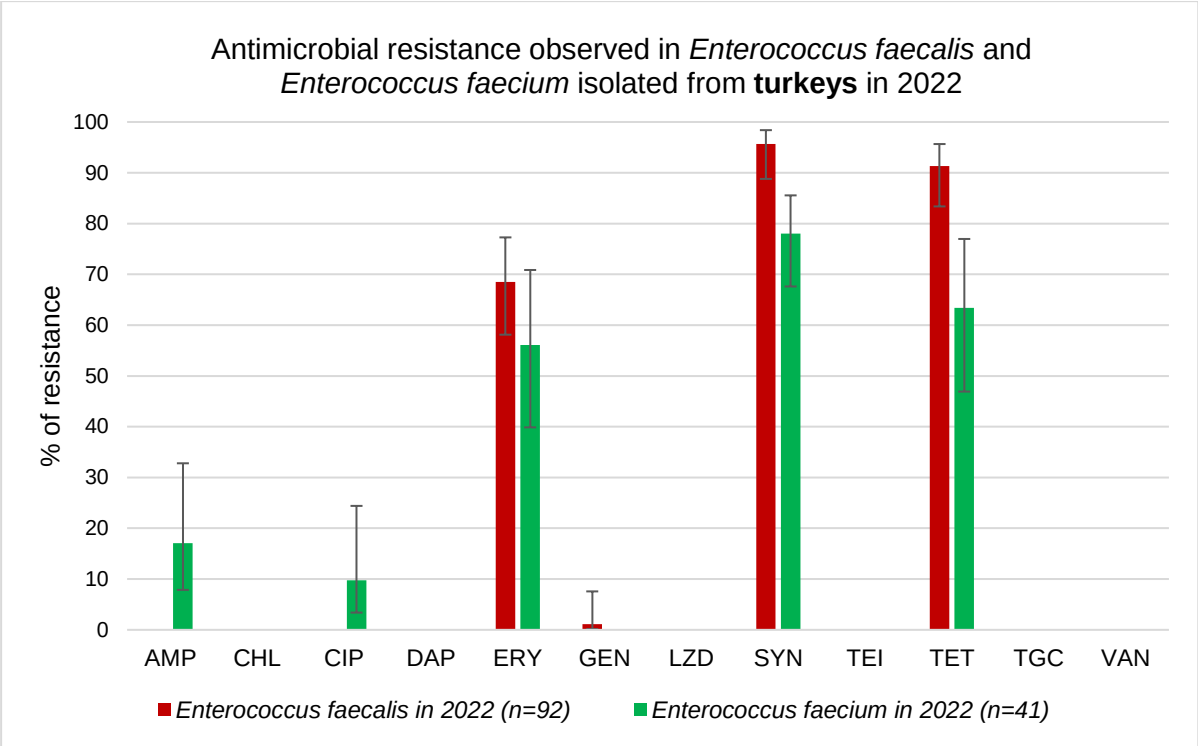


Figure 48. Percentages of antimicrobial resistance in *Enterococcus faecalis* (n=40) and *Enterococcus faecium* (n=92) isolated from turkeys in 2022.

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 204 antimicrobial susceptibility tests were performed after isolation of *Enterococcus faecalis* (n=51) and *Enterococcus faecium* (n=153) from breeder samples.

In breeding hens samples, very high resistance to tetracycline (66.7%) and high resistance to erythromycin (23.5%) were observed in *E. faecalis*. In *E. faecium*, an extremely high rate of resistance to quinupristin/dalfopristin (81.7%) was observed as well as a high rate to tetracycline (45.8%) and a moderate rate to erythromycin (12.4%). A low level of resistance to ampicillin (7.2%) was also observed in *E. faecium* only, this resistance being absent in *E. faecalis*. A low rate of resistance to chloramphenicol was observed in *E. faecium* (1.3%), this resistance was absent in *E. faecalis*. Similarly, a low resistance rate to ciprofloxacin (5.9%) was observed in *E. faecium,* only.

Following the modification of the resistance threshold (from 4 to 8 mg/L) to daptomycin in *E. faecium* in 2021, this resistance was found in a very low rate (0.7%) among strains isolated from breeding hens in 2022. This resistance was absent in *E. faecalis*. One *E. faecium* isolated from breeders showed resistance to linezolid, characterized by a minimum inhibitory concentration of 8 mg/L; and one *E. faecium* showed resistance to tigecycline, characterized by a minimum inhibitory concentration of 0.5 mg/L. No resistance to gentamicin, teicoplanin or vancomycin was observed in breeders in 2022 (see Figure 49).

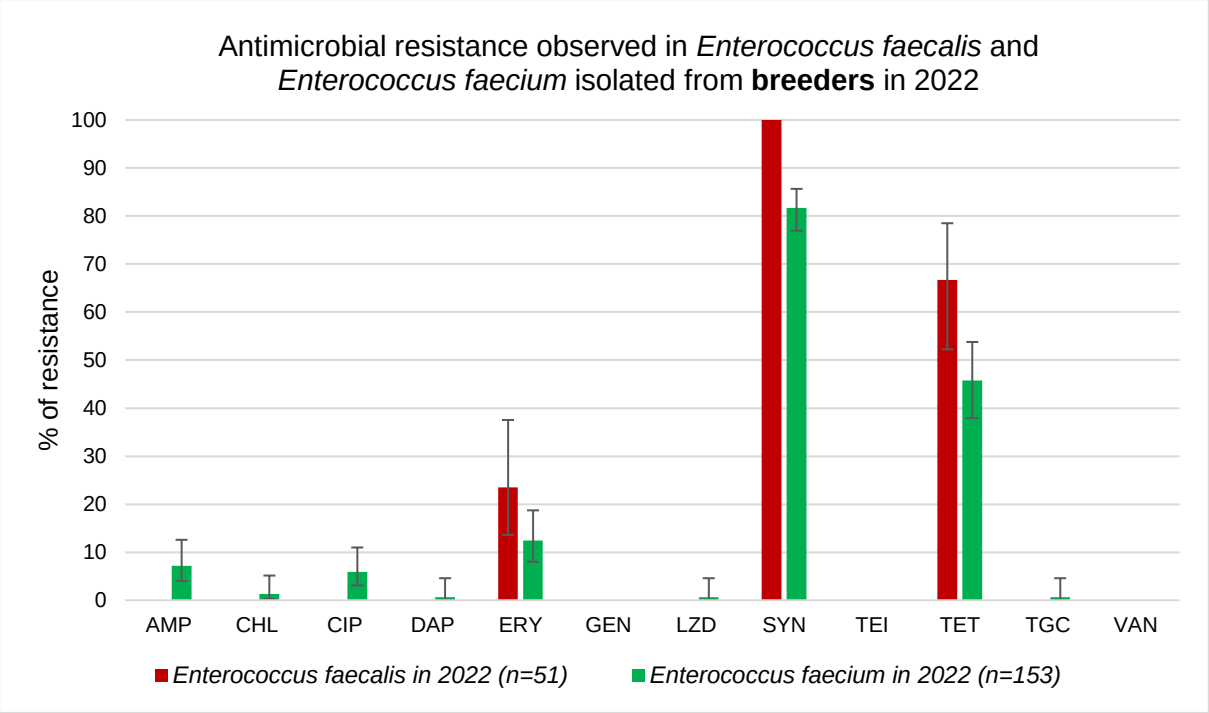


Figure 49. Percentages of antimicrobial resistance in *Enterococcus faecalis* (n=51) and *Enterococcus faecium* (n=153) isolated from breeders in 2022.

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 267 antimicrobial susceptibility tests were performed after isolation of *Enterococcus faecalis* (N=111) and *Enterococcus faecium* (N=156) from laying hens samples.
A very high rate of resistance to tetracycline (51.4%) and high to erythromycin (20.7%) was observed in *E. faecalis*. Within *E. faecium* isolates, a very high rate of resistance to quinupristin/dalfopristin (51.9%), as well as a moderate rate to erythromycin (12.4%) 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 (1.8%). On the contrary, a low level of resistance to ampicillin (2.6%) and a very low level to ciprofloxacin (0.6%) were observed in *E. faecium* only (see Figure 44).
Following the modification of the resistance threshold (from 4 to 8 mg/L) to daptomycin in *E. faecium* in 2021, this resistance was absent from the strains isolated from laying hens in 2022. No resistance to gentamicin, linezolid, teicoplanin, tigecycline or vancomycin was observed in layers in 2022 (see Figure 50).

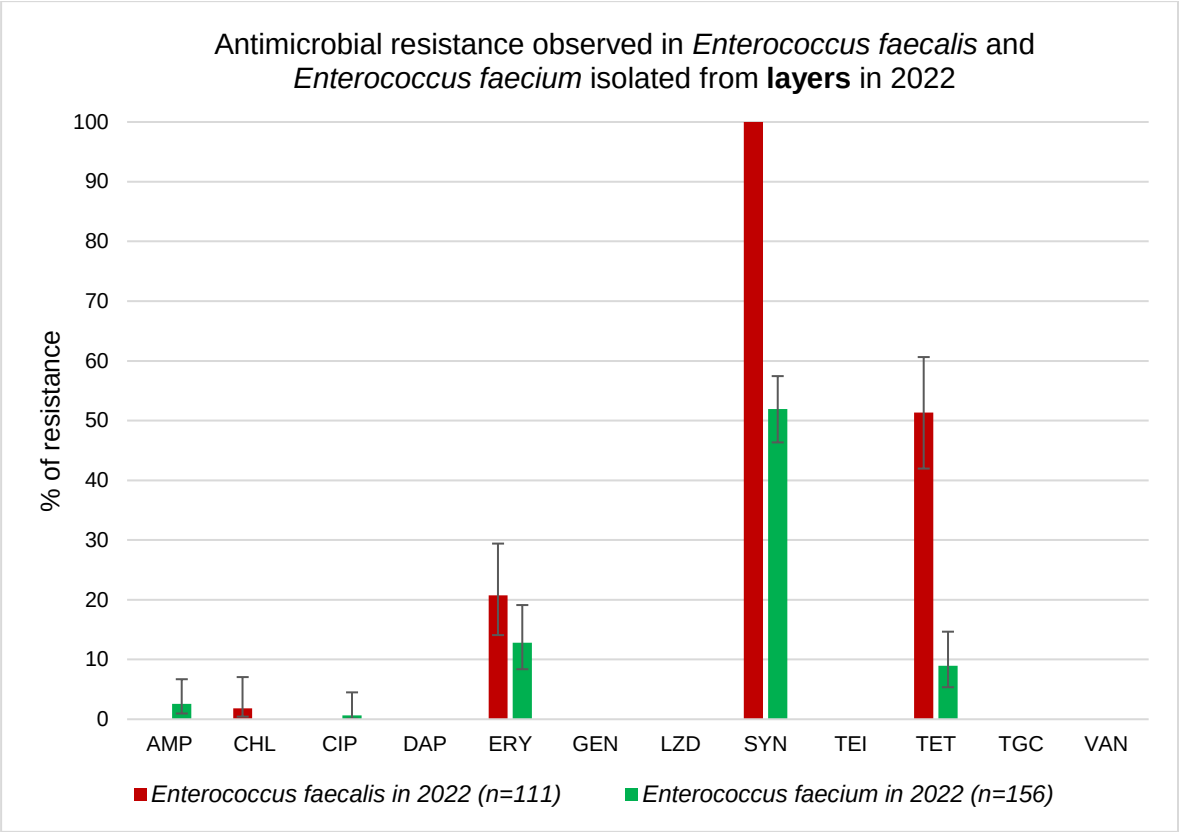


Figure 50. Percentages of antimicrobial resistance in *Enterococcus faecalis* (n=111) and *Enterococcus faecium* (n=156) isolated from layers in 2022.

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).

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

A total of 245 antimicrobial susceptibility tests were performed after isolation of *Enterococcus faecalis* (n=83) and *Enterococcus faecium* (n=162) from veal calve samples.

Within veal calve samples, extremely high resistance rates were observed for tetracycline (94.0%) and erythromycin (78.3%) in *E. faecalis*. Extremely high resistance rates to quinupristin/dalfopristin (82.0%), and high to tetracycline (48.4%) and erythromycin (42.2%) were observed in *E. faecium*. Contrary to a high resistance rate observed in *E. faecalis* (44.6%), resistance to chloramphenicol was low (1.9%) in *E. faecium*.

A high rate of resistance to gentamicin (20.5%) was observed in *E. faecalis* while this resistance was low in *E. faecium* (2. 5%). The resistance rates observed to ciprofloxacin were low in *E. faecalis* and *E. faecium* (1.9% and 6.0 % respectively), as well as the resistance rate to ampicillin (6.2%) observed in *E. faecium* only.

Following the modification of the resistance threshold (from 4 to 8 mg/L) to daptomycin in *E. faecium* in 2021, this resistance was absent from *E. faecium* isolates, as well as from *E. faecalis* isolated from veal calves in 2022. In total, 13 strains (11 *E. faecalis* and 2 *E. faecium*) showed resistance to linezolid, characterized by a minimum inhibitory concentration of 8 mg/L (n=12) or >64 mg/L (n=1). One *E. faecium* strain showed resistance to tigecycline, characterized by a minimum inhibitory concentration of 0.5 mg/L. No resistance to teicoplanin or vancomycin was observed in veal calves in 2022 (see Figure 51).

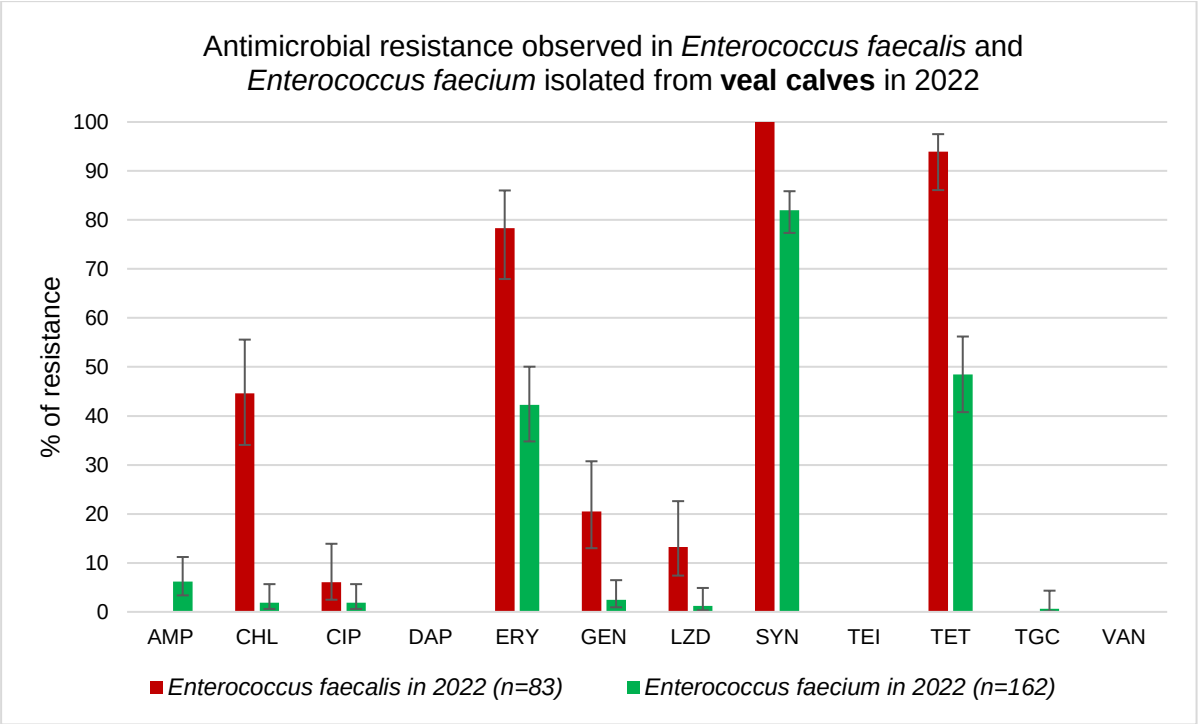


Figure 51. Percentages of antimicrobial resistance in *Enterococcus faecalis* (n=83) and *Enterococcus faecium* (n=162) isolated from veal calves in 2022.

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).

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

A total of 221 antimicrobial susceptibility tests were performed after isolation of *Enterococcus faecalis* (n=51) and *Enterococcus faecium* (n=170) from pig samples.

Within pig samples, a very high rate of resistance to tetracycline (60.8%), and a high rate of resistance to erythromycin (35.3%) were observed in *E. faecalis*. In *E. faecium*, an extremely high percentage of resistance to quinupristin/dalfopristin (82.9%) and high resistance to tetracycline (44.1%) were observed, while the resistance rate to erythromycin was low (7.1%). The observed resistance rate to chloramphenicol was moderate in *E. faecalis* (17.6%) while low (1.2%) in *E. faecium*. A low resistance rate to ampicillin (7.6%) was observed in *E. faecium* and no resistance was observed in *E. faecalis*. In addition, a low rate of resistance to gentamicin (9.8%) was observed in *E. faecalis* whereas this resistance was absent in *E. faecium*. Finally, resistance to ciprofloxacin was absent in *E. faecalis* and found in a low rate (1.8%) in *E. faecium* (see Figure 52).

Following the modification of the resistance threshold (from 4 to 8 mg/L) to daptomycin in *E. faecium* in 2021, this resistance was absent from *E. faecium*, as well as from *E. faecalis* isolated from pigs.

In 2022, 4 strains isolated from pig samples (n=2 *E. faecium* and 2 *E. faecalis*) showed resistance to linezolid, characterized by a minimum inhibitory concentration of 8 mg/L (n=3) or 16 mg/L (n=1). No resistance to teicoplanin, tigecycline or vancomycin was observed in pigs in 2022.

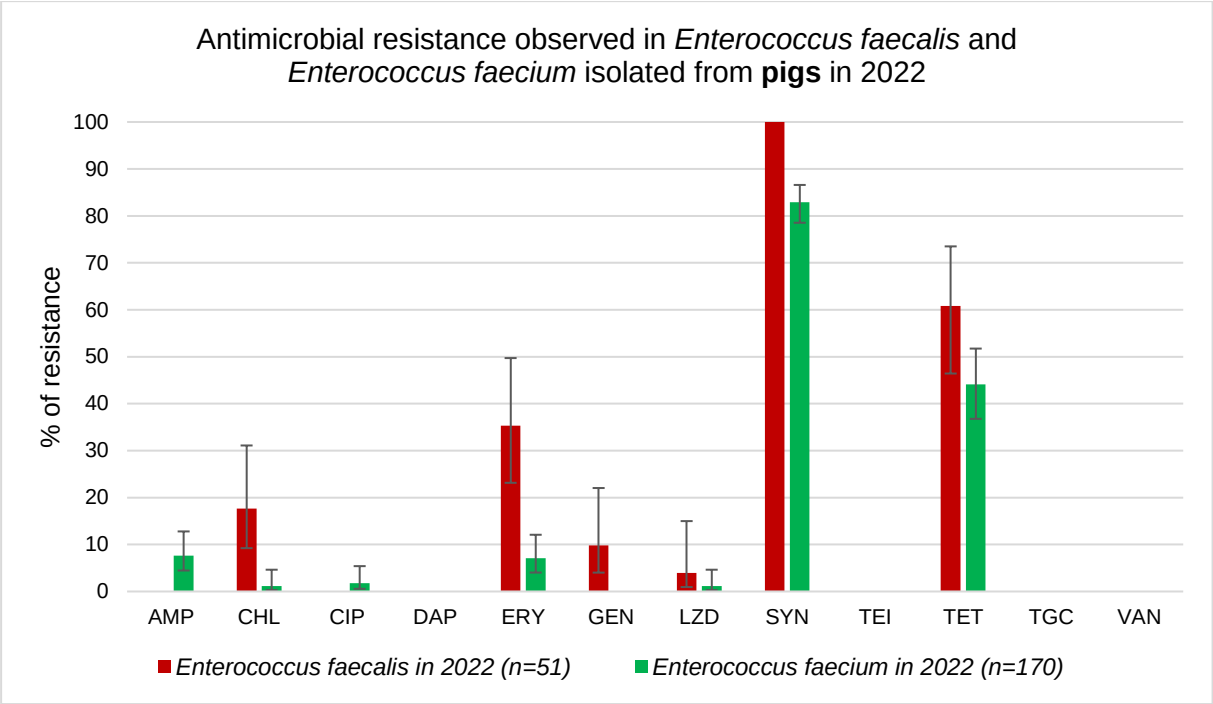


Figure 52. Percentages of antimicrobial resistance in *Enterococcus faecalis* (N=51) and *Enterococcus faecium* (N=170) isolated from pigs in 2022.

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).

3.2.11.5. Comparison of antimicrobial resistances observed in *Enterococcus faecalis* and *Enterococcus faecium* per animal matrix between 2019 and 2022

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 53 to Figure 58), with the exception of significant decreases in the rate of resistance to certain antimicrobials observed in 2022.

Indeed, the resistance rate to chloramphenicol has significantly decreased by 18.0% (from 62.6% to 44.6%) since 2019 in *E. faecalis* isolated from veal calves (see Figure 58). In *E. faecium* isolated from broilers, a significant decrease of 17.7% (29.0% to 11.3%) in ampicillin resistance was observed in 2022 compared to 2019 (see Figure 53). In 2022, the resistance rate to daptomycin was very low (0.7%) in *E. faecium* isolated from turkeys, and this resistance was absent from all the remaining 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 was observed during the studied years.

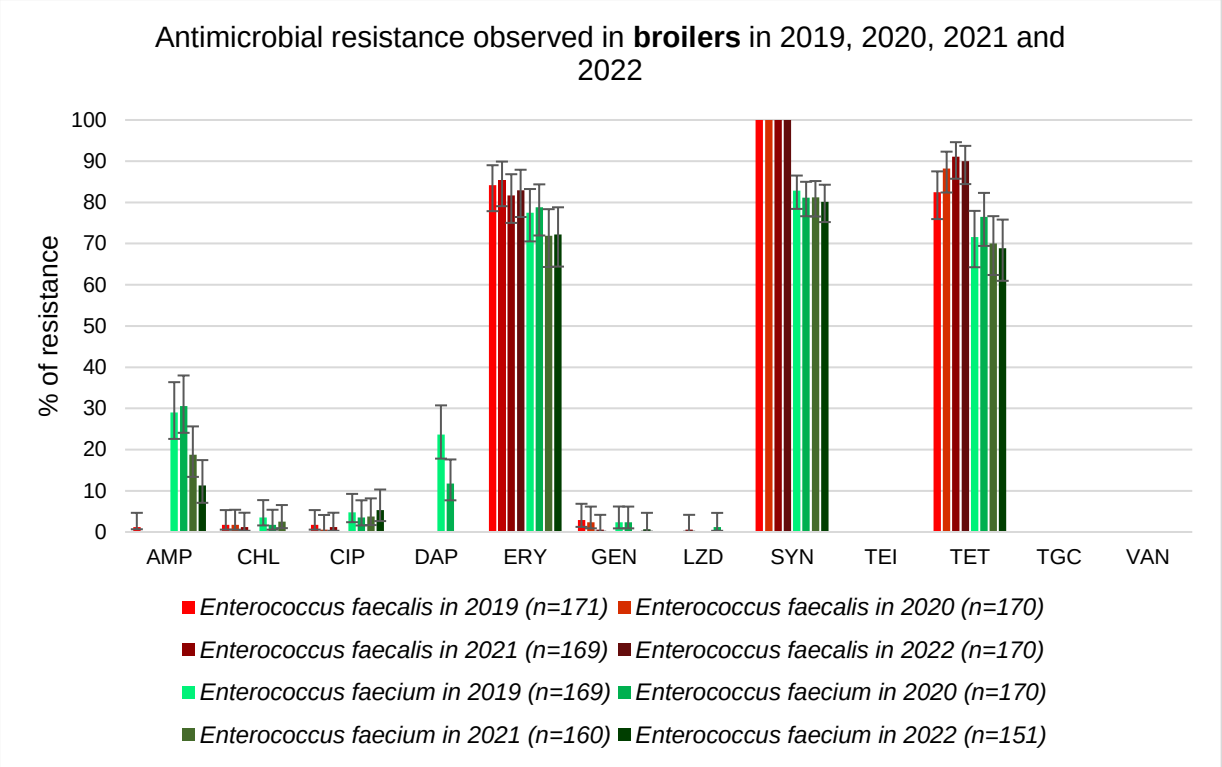


Figure 53. Percentages of antimicrobial resistance in *Enterococcus faecalis* and *Enterococcus faecium* isolated from broilers from 2019 to 2022.

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).

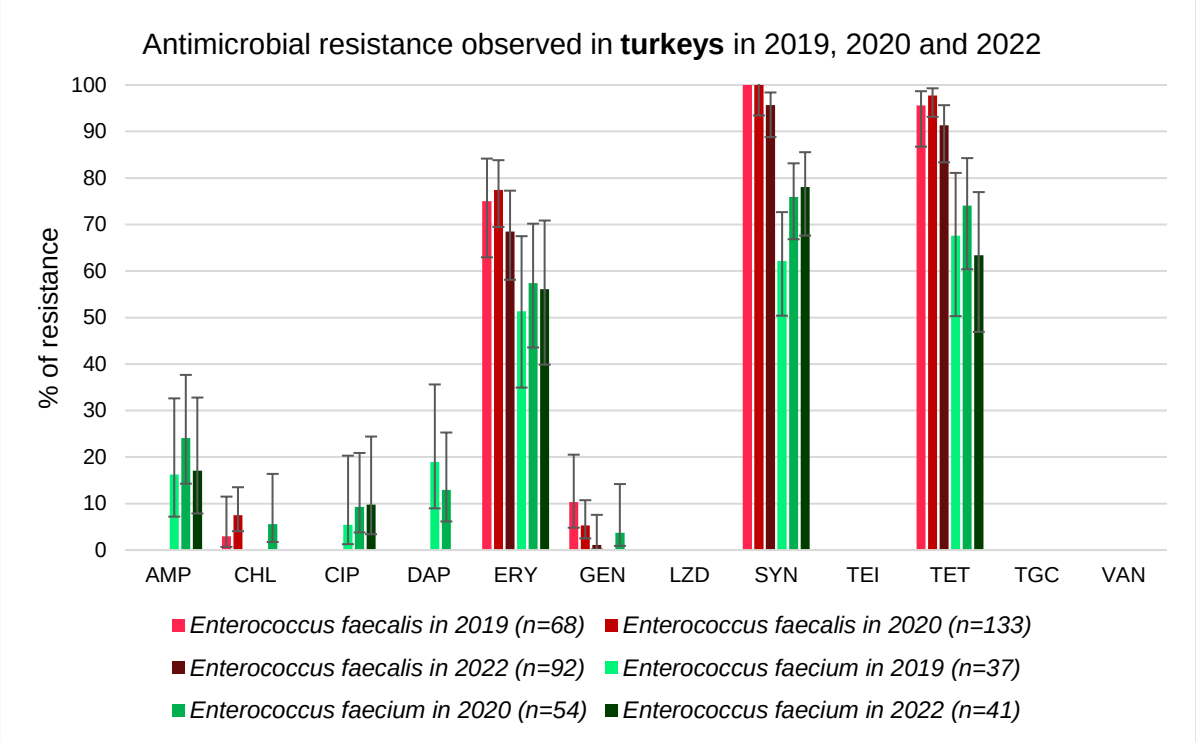


Figure 54. Percentages of antimicrobial resistance in *Enterococcus faecalis* and *Enterococcus faecium* isolated from turkeys from 2019 to 2022.

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).

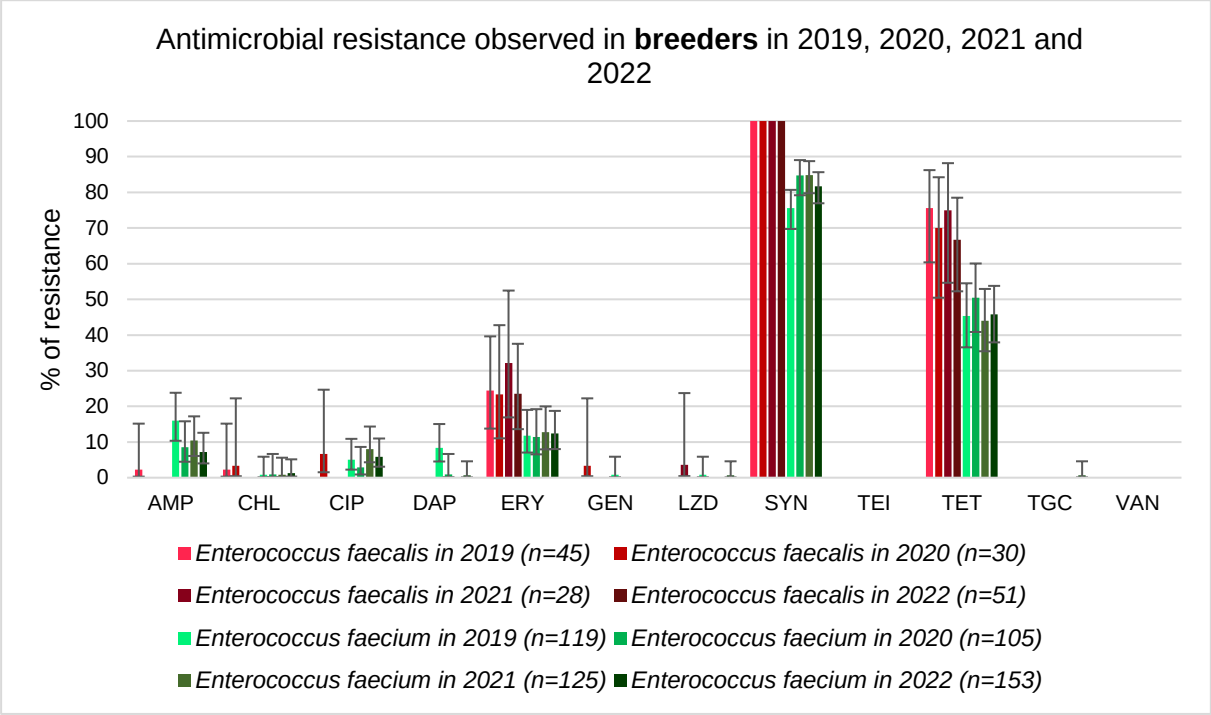


Figure 55. Percentages of antimicrobial resistance in *Enterococcus faecalis* and *Enterococcus faecium* isolated from breeders from 2019 to 2022.

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).

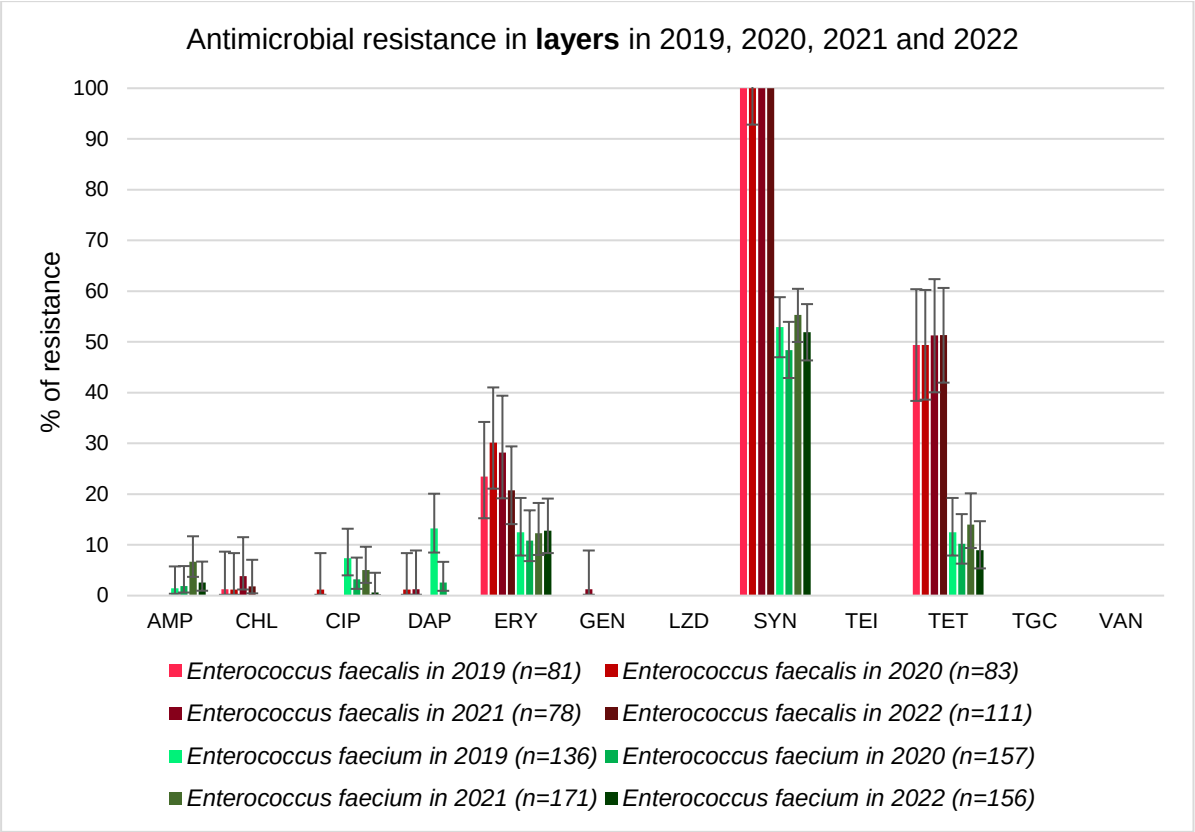


Figure 56. Percentages of antimicrobial resistance in *Enterococcus faecalis* and *Enterococcus faecium* isolated from layers from 2019 to 2022.

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).

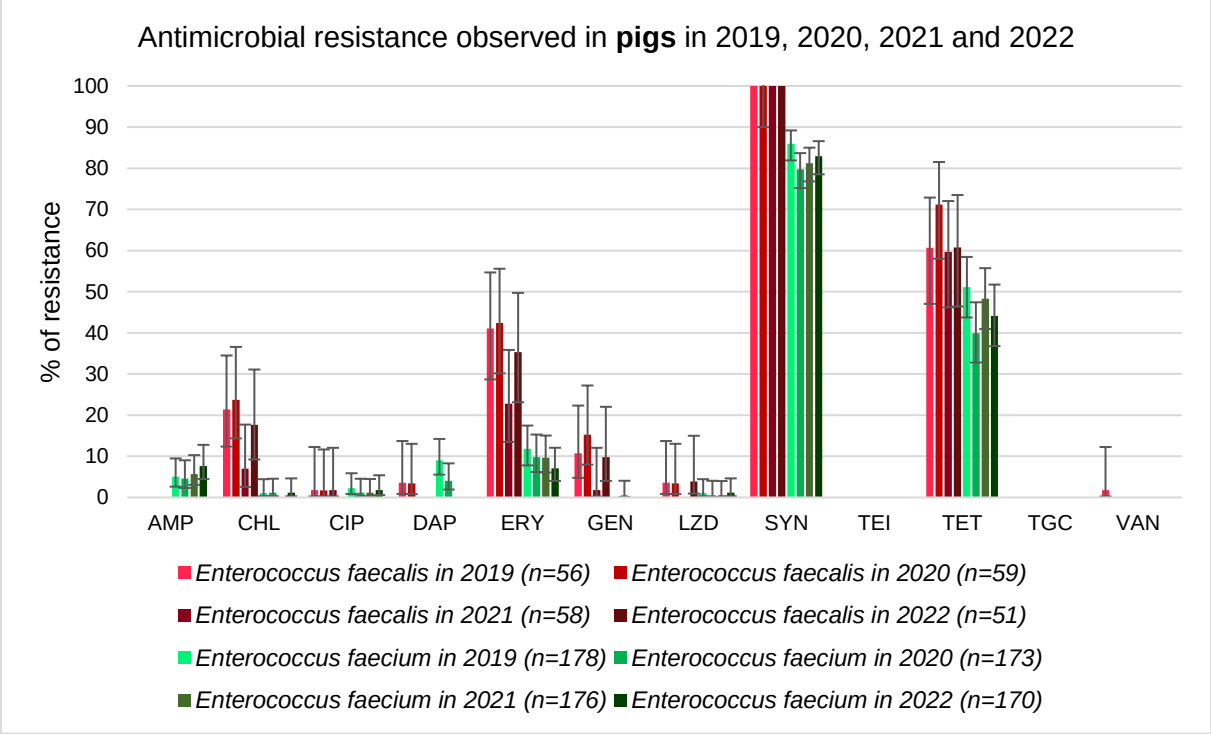


Figure 57. Percentages of antimicrobial resistance in *Enterococcus faecalis* and *Enterococcus faecium* isolated from pigs from 2019 to 2022.

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).

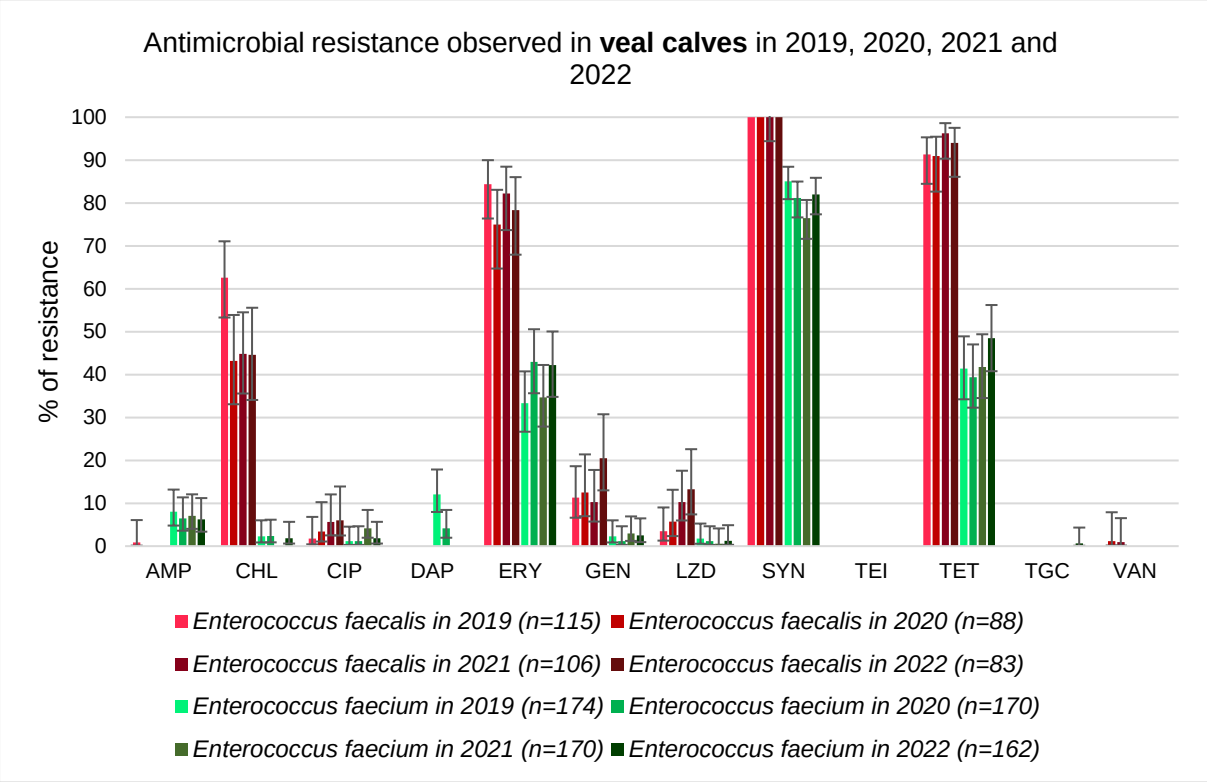


Figure 58. Percentages of antimicrobial resistance in *Enterococcus faecalis* and *Enterococcus faecium* isolated from veal calves from 2019 to 2022.

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).

3.2.11.6. 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 2022, the highest number of multi-resistant strains was observed in veal calves (60.2% of *E. faecalis*) and in broilers and turkeys (60.3% and 47.5% of *E. faecium,* respectively) (see Figure 60 and Figure 62). *E. faecalis* isolated from broilers were mostly resistant to 1 (17.6%) or 2 (77.6%) antimicrobials, while 18.5%, 12.6%, 47.0%, 12.6% and 0.6% of *E. faecium* were resistant to 1, 2, 3, 4 or 5 antimicrobials respectively. Similarly, *E. faecalis* isolated from turkeys were mostly resistant to 1 (29.3%), 2 (64.1%) or 3 (1.1%) antimicrobials, while 17.5%, 25.0%, 30.0%, 15.0% and 2.5% were resistant to 1, 2, 3, 4 or 5 antimicrobials. Among poultry, the strains isolated from breeding hens and laying hens were globally less resistant. Indeed, 43.1% and 35.1% of *E. faecalis* and 38.6% and 43.6% of *E. faecium* resistant to 1 antimicrobial as well as 23.5% and 18.0% of *E. faecalis* and 34.0% and 14.7% of *E. faecium* resistant to 2 different antimicrobials, respectively. Within pig samples, 33.3% of *E. faecalis* were resistant to 1, 11.8% to 2, 15.7% to 3 and 5.9% to 4 different antimicrobials. Among *E. faecium* isolated from pigs, 41.8% were resistant to 1 antimicrobial, 35.9% to 2, 8.8% to 3 and 1.2% to 5 antimicrobials. In veal calves, *E. faecalis* were resistant to 1 (15.7%), 2 (18.1%), 3 (41.0%), 4 (15.7%), 5 (2.4%) and 6 (1.2%) different antimicrobials. Similarly, 43.8%, 29.0%, 17.3%, 6.2%, and 1.2% of *E. faecium* isolated from

veal calves were resistant to 1, 2, 3, 4 and 6 antimicrobials respectively. Strains isolated from veal calves showed the greatest number of resistances, with 1.2% of *E. faecium* and 1.2% of *E. faecalis* resistant to 6 different antimicrobials (see Figure 59, Figure 61, Table 18 and Table 19).

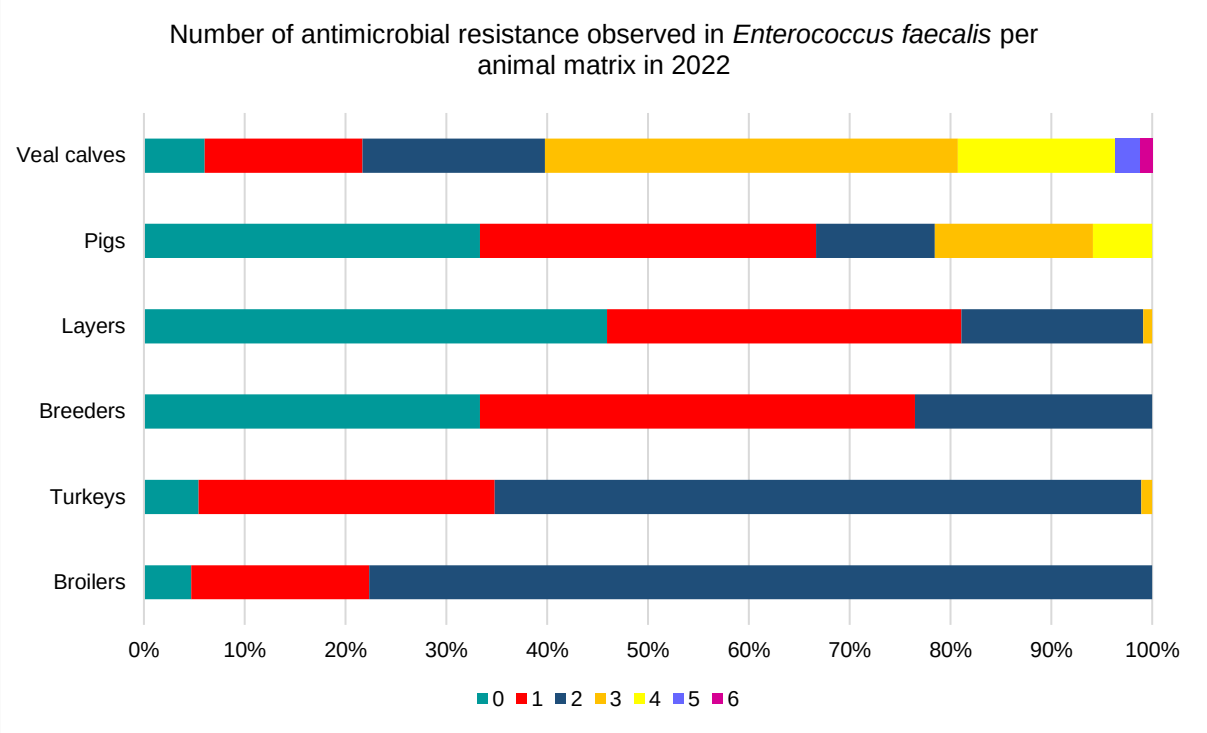


Figure 59. Percentages of *Enterococcus faecalis* according to the number of resistance and the animal matrix in 2022.

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.

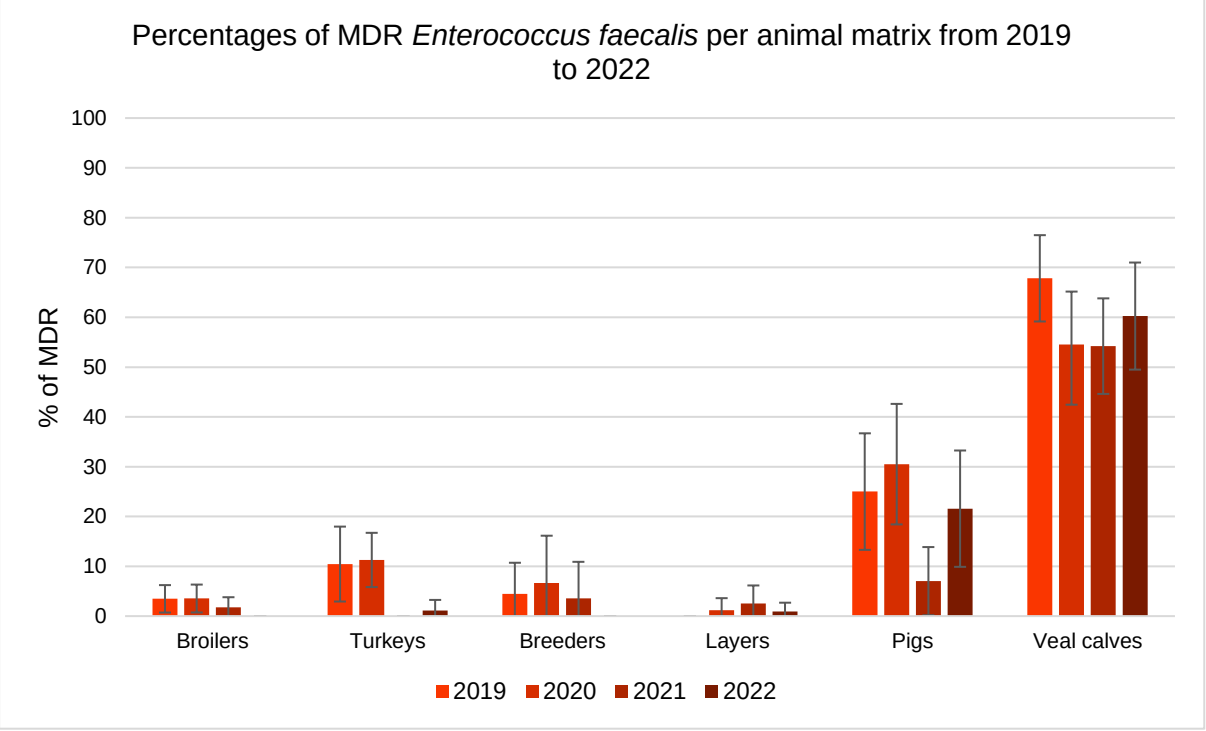


Figure 60. Percentages of MDR *Enterococcus faecalis* observed per animal matrix between 2019 and 2022.

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. No data were collected from turkeys in 2021.

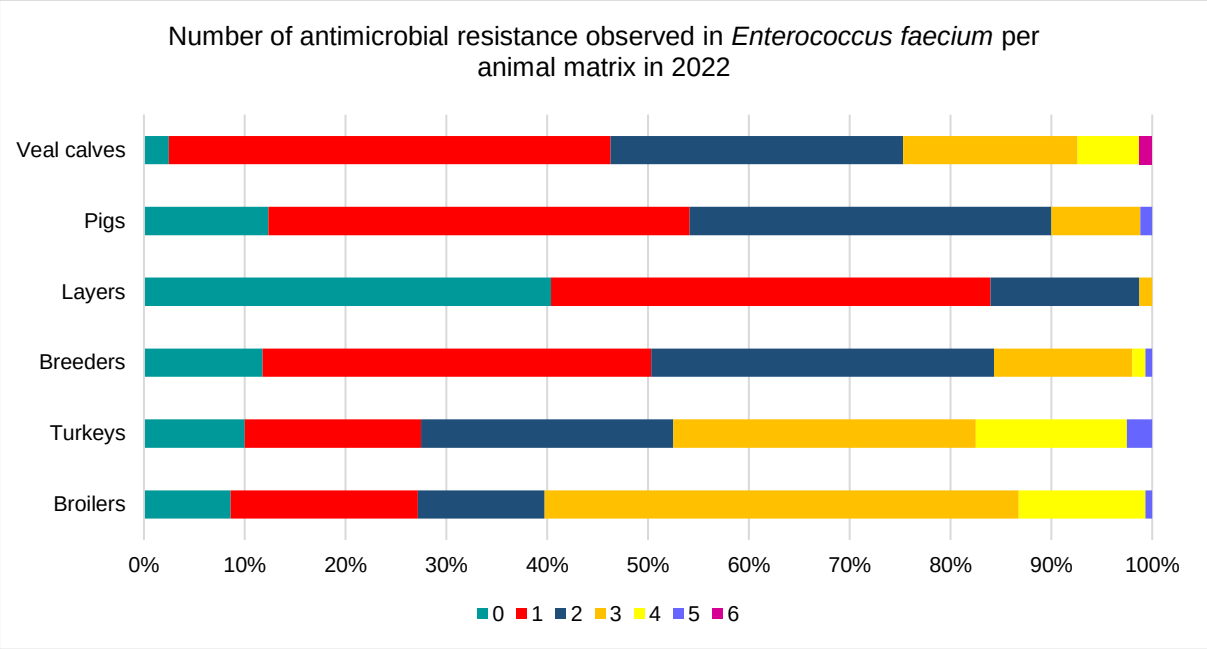


Figure 61. Percentages of *Enterococcus faecium* according to the number of resistance and the animal matrix in 2022.

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.

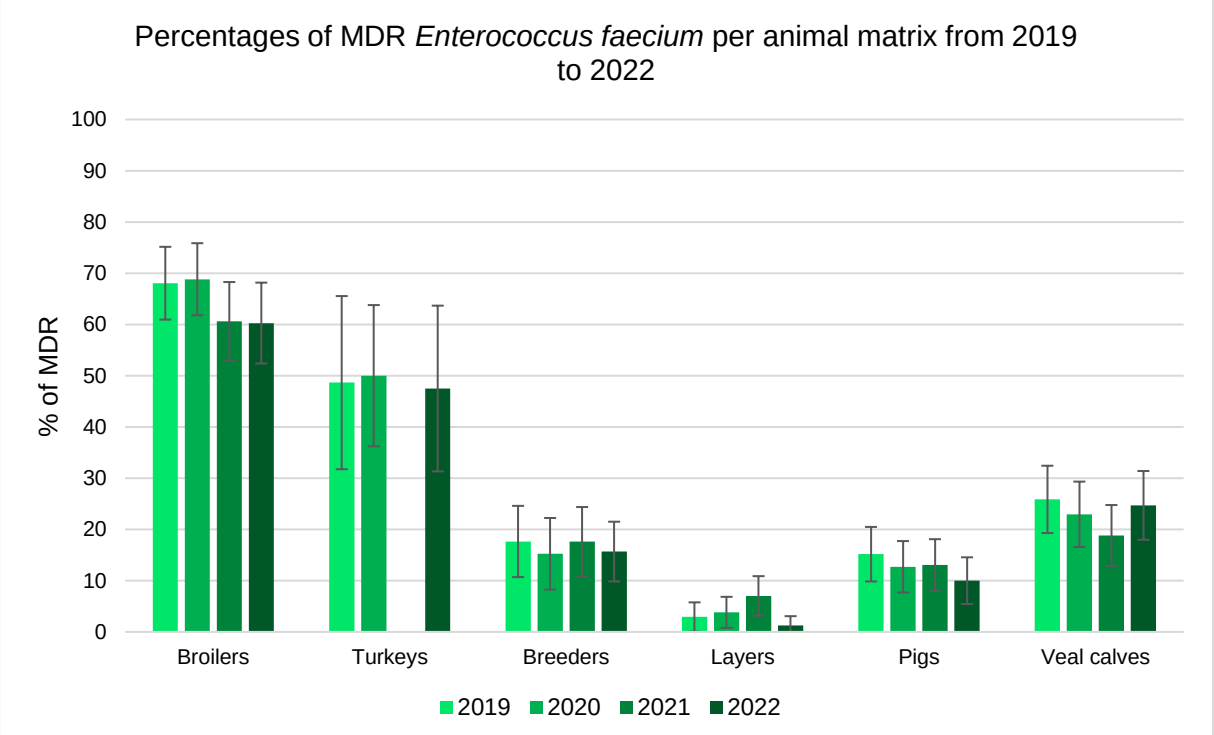


Figure 62. Percentages of MDR *Enterococcus faecium* observed per animal matrix between 2019 and 2022.

A strain is considered multi-resistant when it is resistant to at least 3 different families of antibiotics. No data were collected from turkeys in 2021.

MDR	Resistance profiles	Broilers (n=170)	Turkeys (n=92)	Breeders (n=51)	Layers (n=111)	Pigs (n=51)	Veal calves (n=83)
6	CHL CIP ERY GEN LZD TET	-	-	-	-	-	1
5	CHL CIP ERY GEN TET	-	-	-	-	-	1
	CIP ERY GEN LZD TET	-	-	-	-	-	1
4	CHL CIP ERY TET	-	-	-	-	-	2
	CHL ERY GEN TET	-	-	-	-	2	4
	CHL ERY LZD TET	-	-	-	-	1	7
3	CHL ERY TET	-	-	-	1	5	22
	ERY LZD TET	-	-	-	-	1	2
	ERY GEN TET	-	1	-	-	2	10
2	CHL ERY	-	-	-	-	1	-
	CHL TET	-	-	-	1	-	-
	ERY TET	132	59	12	19	4	15
	GEN TET	-	-	-	-	1	-
1	ERY	9	3	-	2	2	-
	TET	21	24	22	37	15	13

Table 18. List of phenotypic resistance profiles observed in *Enterococcus faecalis* isolates and classified by animal matrix in 2022.

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*.

MDR	Resistance profiles	Broilers (n=160)	Turkeys (n=40)	Breeders (n=153)	Layers (n=156)	Pigs (n=170)	Veal calves (n=161)
6	AMP CHL CIP ERY SYN TET	-	-	-	-	-	1
	ERY GEN LZD SYN TET TGC	-	-	-	-	-	1
5	AMP CHL LZD SYN TET	-	-	-	-	1	-
	AMP CIP ERY SYN TET	1	1	-	-	-	-
	CHL ERY LZD SYN TET	-	-	-	-	1	-
	CIP DAP SYN TET TGC	-	-	1	-	-	-
4	AMP CIP ERY SYN	-	1	-	-	-	-
	AMP ERY SYN TET	15	4	1	-	-	5
	CHL ERY LZD SYN	-	-	1	-	-	1
	CIP ERY SYN TET	3	1	-	-	-	1
	ERY GEN SYN TET	1	-	-	-	-	3
3	AMP ERY TET	1	-	-	-	-	-
	AMP SYN TET	-	-	5	-	8	4
	CHL ERY TET	-	-	1	-	-	1
	CIP ERY SYN	3	-	-	-	-	-
	CIP SYN TET	1	-	4	1	-	-
	ERY SYN TET	66	12	11	1	7	23
2	AMP SYN	-	-	5	3	3	-

	AMP TET	-	1	-	1	1	-
	CIP SYN	-	1	4	-	-	-
	CIP TET	-	-	-	-	-	1
	ERY SYN	6	3	3	14	2	13
	ERY TET	8	1	1	2	2	18
	SYN TET	5	4	39	3	53	15
1	CIP	-	-	-	-	3	-
	ERY	5	-	1	3	-	1
	SYN	20	5	51	59	66	66
	TET	3	2	7	6	2	4

Table 19. List of phenotypic resistance profiles observed in *Enterococcus faecium* isolates and classified by animal matrix in 2022.

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.

3.2.11.7. Investigation by NGS of *Enterococcus faecalis* and *Enterococcus faecium* isolated in 2022

- Selection criteria

In 2022, 19 enterococci (13 *E. faecalis* and 6 *E. faecium*) isolated from breeding hens (n=2), pigs (n=4) et veal calves (n=13) were investigated by sequencing. Each strain was selected according to its phenotypic profile, independently of the animal matrix and presented at least one of the following criteria: resistance to linezolid and/or resistance to tigecycline. No phenotypic resistance to vancomycin was observed in *Enterococcus* in 2022.

- 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 9 STs were observed only once, 2 STs (ST 19 and 480) observed in 2 different strains, and 2 STs (ST 40 and 314) observed in 3 strains (isolated from veal calves); and some of which had the same phenotype (see Table 20). 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 some of these strains.

Indeed, the comparison of the 2 *E. faecalis* (VAR 917 and VAR 1079) isolated from veal calves characterized by an ST19 with a same resistance phenotype (see Table 20) showed that these strains were definitely related (no allelic difference observed). Similarly, the 3 *E. faecalis* (VAR 918, VAR 1059 and VAR 1063) isolated from veal calves characterized by an ST314 and showing a same resistance phenotype were very likely related. Although presenting different phenotypic resistance profiles, the 2 *E. faecalis* (VAR 1062 and VAR 1076) isolated from veal calves and belonging to ST480 were very likely related. Also, among the 3 *E. faecalis* (VAR 920, VAR 921 and VAR 1060) isolated from veal calves and belonging to ST40, two of them (VAR 920 and VAR 921) were definitely related, and are both likely related to the third strain (VAR 1060).

Table 20. List of enterococci sequenced by NGS in 2022 and their phenotypic profile obtained from the antimicrobial susceptibility study.

Sciensano VAR ID	Species	Phenotype	ST	Animal matrix
VAR-920	*E. faecalis*	ERY LZD TET	40	veal calves
VAR-921	*E. faecalis*	ERY LZD TET	40	veal calves
VAR-916	*E. faecalis*	ERY LZD TET	915	pigs
VAR-917	*E. faecalis*	CHL ERY LZD TET	19	veal calves
VAR-1079	*E. faecalis*	CHL ERY LZD TET	19	veal calves

VAR-1073	*E. faecalis*	CHL ERY LZD TET	21	veal calves
VAR-1060	*E. faecalis*	CHL ERY LZD TET	40	veal calves
VAR-918	*E. faecalis*	CHL ERY LZD TET	314	veal calves
VAR-1059	*E. faecalis*	CHL ERY LZD TET	314	veal calves
VAR-1063	*E. faecalis*	CHL ERY LZD TET	314	veal calves
VAR-991	*E. faecalis*	CHL ERY LZD TET	409	pigs
VAR-1062	*E. faecalis*	CIP ERY GEN LZD TET	480	veal calves
VAR-1076	*E. faecalis*	CHL CIP ERY GEN LZD TET	480	veal calves
VAR-1061	*E. faecium*	CHL ERY LZD SYN	8	breeding hens
VAR-992	*E. faecium*	CHL ERY LZD TET	104	veal calves
VAR-990	*E. faecium*	CHL ERY LZD SYN TET	5	pigs
VAR-1078	*E. faecium*	CIP DAP SYN TET TGC	10	breeding hens
VAR-919	*E. faecium*	AMP CHL LZD SYN TET	140	pigs
VAR-1077	*E. faecium*	ERY GEN LZD SYN TET TGC	55	veal calves

VAR-ID : internal number for NGS analysis. 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 **93.3**% (n=83/89) of individual observed resistance phenotypes (see Table 21).

Except one, all linezolid-resistant strains analyzed (n=18) were characterized by the presence of the *optrA* gene (n=16 with 12 isolated from veal calves, 3 from pigs and 1 from breeding hens) or the combination of *optrA*/*poxtA* (n=1 isolated from veal calve). 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), *msr(C)* (n=1), *erm(A)/msr(C)* (n=1) and *erm(A)/erm(B)/msr(C)* (n=2). Of 13 strains resistant to chloramphenicol, 12 carried at least one resistance gene, namely *fexA* (n=8), *cat/fexA* (n=2) and *catp*$_{C221}$*/fexA* (n=2). No gene was detected in the last resistant strain. Five strains phenotypically susceptible to chloramphenicol also carried resistance genes such as *fexA* (n=3), *fexB* (n=1) and *cat/fexA* (n=1). The presence of resistance genes in chloramphenicol-susceptible isolates may be explained by the inducibility of these genes (Kowalewicz *et al.*, 2022, Schwarz *et al.,* 2016). Resistance to quinupristin/dalfopristin (Q/D, SYN) was characterized by the presence of the *lsaA* (n=11) or *lsaA/lsaE* (n=2) genes in *E. faecalis* (n= 13) and by the presence of *msr(C)* (n=3) or *lsa(E)/msr(C)* (n=1) in *E. faecium* (n=5). No gene was found in in the last resistant *E. faecium*. The *msrC* gene was also found in one Q/D-susceptible *E. faecium* and in one erythromycin-susceptible *E. faecium*, 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 2 out of 3 of the resistant strains investigated by NGS. No genes were detected in the last gentamicin-resistant strain. All tetracycline resistant strains sequenced (n=18) carried at least one resistance gene, namely *tet(M)* (n=3) or the combination of 2 genes *tet(L)/tet(M)* (n=15). No gene has been identified explaining the ampicillin resistance observed in one sequenced *E. faecium*. However, this resistance in *E. faecium* may be due to increased production of PBP5 following the presence of mutations (pbp5 p.V24A, pbp5 p.S27G, pbp5 p.R34Q, pbp5 p.G66E, pbp5 p.E100Q, pbp5 p.K144Q, pbp5 p.T172A, pbp5 p.L177I, pbp5 p.A216S, pbp5 p.T324A, pbp5 p.N496K, pbp5 p.A499I, pbp5 p.E525D) in the beta subunit of this protein, which was observed in the resistant isolate. Resistance to ciprofloxacin, and to fluoroquinolones in general, is characterized by the presence of mutations in the *parC* and *gyrA* genes and widely described in gram-negative patients. Mutations were observed in our 3 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)), *parC* p.A391V (n=1), *gyrA* p.S83Y (n=2, also described in *Salmonella* spp. (Farrera *et al.,* 2018)) and *gyrA* p.D759N (n=2). Except for one *E. faecium* isolated from turkeys, no resistance to daptomycin was

observed in 2022. This absence of resistance could be related to the modification of the threshold of this resistance within *E. faecium* specie (from 4 to 8 mg/L) in 2021. In enterococci, resistance to daptomycin is likely to be associated with mutations in intrinsic genes, such as *liaFSR, cls* or *gdpD* (Bender *et al*, 2020). However, none of them have been detected in our resistant isolate. In addition to that, mechanisms leading to this resistance are not yet well understood.
In addition, no resistance genes were detected in the two *E. faecium* isolates (n=1 from breeder, n=1 from veal calve) exhibiting resistance to tigecycline. Besides, the presence of mutations in genes such as *tet(L), tet(M)* or *rpsJ* has been also reported to play a role in resistance and/or decreased susceptilibity to tigecycline (Bender *et al*, 2020, Fiedler *et al.,* 2016). These mutations are not yet targeted by the current AMR detection tools and more investigation would be needed to characterize the genetic background of the phenotypical tigecycline resistance in these isolates.

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).

In addition to antimicrobial resistance genes, sequencing of enterococci revealed the presence of the *clpL* gene in 3 *E. faecalis* isolated from veal calves. Indeed, this gene codes for a protease reported in the literature to be widely present in Gram-positive bacteria (*L. monocytogenes*, *Streptococcus*) and to be involved in many mechanisms such as resistance to stress, disinfectants or antibiotics (Jana *et al*., 2021; Pöntinen *et al*., 2017). Also, to our knowledge, *clpL* has not yet been described in *Enterococcus* spp.

Table 21. List of resistance genes identified by NGS per resistant phenotype observed in enterococci in 2022.

Sciensa-no ID	Specie	Animal matrix	CHL	ERY	GEN	LZD	SYN	TET	TGC
VAR-916	*E. faecalis*	pigs	*cat, fexA*	*erm(A)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*	
VAR-917	*E. faecalis*	veal calves	*fexA*	*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*	
VAR-918	*E. faecalis*	veal calves	*fexA*	*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*	
VAR-920	*E. faecalis*	veal calves	*fexA*	*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*	
VAR-921	*E. faecalis*	veal calves	*fexA*	*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*	
VAR-991	*E. faecalis*	pigs	*cat, fexA*	*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*	
VAR-1059	*E. faecalis*	veal calves	*fexA*	*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*	
VAR-1060	*E. faecalis*	veal calves	$catp_{C221}$, *fexA*	*erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*	
VAR-1062	*E. faecalis*	veal calves	*fexA*	*erm(B)*	-	*optrA*	*lsa(A), lsa(E)*	*tet(L), tet(M)*	
VAR-1063	*E. faecalis*	veal calves	*fexA*	*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*	
VAR-1073	*E. faecalis*	veal calves	$catp_{C221}$, *fexA*	*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*	
VAR-1076	*E. faecalis*	veal calves	*cat, fexA*	*erm(B)*	*aac(6')-aph(2'')*	*optrA*	*lsa(A), lsa(E)*	*tet(L), tet(M)*	
VAR-1079	*E. faecalis*	veal calves	*fexA*	*erm(A), erm(B)*		*optrA*	*lsa(A)*	*tet(L), tet(M)*	
VAR-919	*E. faecium*	pigs	*fexA*	*msr(C)*		*optrA*	*msr(C)*	*tet(M)*	
VAR-990	*E. faecium*	pigs	-	*msr(C)*		-	*msr(C)*	*tet(M)*	
VAR-992	*E. faecium*	veal calves	*fexA*	*erm(A), erm(B), msr(C)*		*optrA*	*msr(C)*	*tet(L), tet(M)*	
VAR-1061	*E. faecium*	breeding hens	*fexA*	*erm(A), msr(C)*		*optrA*	*msr(C)*		

VAR-1077	*E. faecium*	veal calves	*fexB*	*erm(A), erm(B), msr(C)*	*aac(6')-aph(2'')*	*optrA, poxtA*	*msr(C), lsa(E)*	*tet(L), tet(M)*	-
VAR-1078	*E. faecium*	breeding hens					-	*tet(M)*	-

VAR-ID : internal number for NGS analysis. Each yellow box corresponds to the presence of phenotypic resistance to the antimicrobial cited, with the following antimicrobials: chloramphenicol (CHL), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), quinupristin/dalfopristin (synercid, SYN), tetracycline (TET) and tigecycline (TGC). Each gene found by NGS is indicated below each antimicrobial to which it confers resistance. The genes present in the white boxes are resistance genes observed in strains whose phenotype for the corresponding antibiotic was susceptible. The presence of a "-" dash indicates a resistant phenotype for which no resistance gene was detected by NGS.

- 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 22), regardless of the animal origin of these strains as described elsewhere (Jimenez *et al*., 2013; Rathnayake *et al*., 2012). All strains investigated by NGS in 2022 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 2022. The aggregation substance protein *agg*, which is associated with greater virulence and higher frequency of their presence in clinical strains, was detected in 6 *E. faecalis* on the 19 investigated isolates.

Table 22. List of virulence factors identified by NGS in enterococci in 2022.

VAR ID	Espèce	efaA	ElrA	StrA	Acm/ Ace	ccf/cob/cad			camE	agg	tpx	ebp			hyl		gelE	fsr
VAR-920 VAR-921 VAR-1060	*E. faecalis*	efaAfs	ElrA	SrtA	ace	cCF10	cOB1	cad	camE	agg	tpx	ebpA	ebpB	ebpC	hylA	hylB	gelE	fsrB
VAR-918 VAR-991 VAR-1059 VAR-1063	*E. faecalis*	efaAfs	ElrA	SrtA	ace	cCF10	cOB1	cad	camE	-	tpx	ebpA	ebpB	ebpC	hylA	hylB	gelE	fsrB
VAR-1073	*E. faecalis*	efaAfs	ElrA	SrtA	ace	cCF10	cOB1	cad	camE	agg	tpx	ebpA	ebpB	ebpC	hylA	hylB	gelE	-
VAR-916 VAR-917 VAR-1079	*E. faecalis*	efaAfs	-	SrtA	ace	cCF10	cOB1	cad	camE	-	tpx	ebpA	ebpB	ebpC	-	hylB	gelE	fsrB
VAR-1062 VAR-1076	*E. faecalis*	efaAfs	ElrA	SrtA	ace	cCF10	cOB1	cad	camE	agg	tpx	ebpA	ebpB	ebpC	hylA	-	-	-
VAR-919 VAR-990 VAR-1077 VAR-1078	*E. faecium*	efaAfm	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VAR-992 VAR-1061	*E. faecium*	efaAfm	-	-	acm	-	-	-	-	-	-	-	-	-	-	-	-	-

VAR-ID : internal number for NGS analysis.

3.2.11.8. Discussion

During enterococci monitoring in 2022, 1292 MALDI-TOF identification tests were performed from 1296 samples taken from poultry, pigs and veal calves, with the remaining 4 samples showing no growth of presumptive enterococci. *Enterococcus faecium* was more frequently isolated than *Enterococcus faecalis* within the samples of breeding hens (85.6%), laying hens (69.9%), veal calves (64.0%) and pigs (66.4%). Conversely, *E. faecalis* was isolated more frequently than *E. faecium* in broiler (65.4%) and turkey (92.0%) samples. The prevalences of bacterial species by animal category observed in 2022 were similar to those observed in previous years. After identification of the bacterial species, 558 antimicrobial susceptibility tests in *Enterococcus faecalis* as well as 832 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 2022. In 2022, 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. Despite a high remaining rate, a significant decrease in chloramphenicol resistance rate was observed in *E. faecalis* isolated from veal calves (-18.0%) in 2022 compared to 2019. A significant decrease of ampicillin resistance (-17.7%) was also observed in *E. faecium* isolated from broilers in 2022, in comparison to 2019.
Some resistance to antimicrobials critically important for human health was also observed in 2022. Resistance to linezolid was observed in 18 strains, namely 13 *E. faecalis* (11 isolated from veal calves and 2 isolated from pigs) and 5 *E. faecium* (2 isolated from veal calves, 2 isolated from pigs and 1 isolated from breeders). Additionally, 2 *E. faecium* isolated from veal calves (n=1) and breeders (n=1) were resistant to tigecycline, also the first time this resistance has been observed since 2019. In 2022, a very low level of resistance to daptomycin has been observed in *E. faecium*, which could be related to the modification of the resistance threshold (from 4 to 8 mg/L) in 2021. No resistance to vancomycin was observed in 2022 and no resistance to teicoplanin was observed during the period 2019-2022.

In general, a greater number of multidrug-resistant strains was observed in veal calves (60.2% of *E. faecalis*) and in broilers and turkeys (60.3% and 47.5% of *E. faecium*, respectively). While already low, the rates of multidrug-resistant *E. faecalis* observed in broilers and turkeys have significantly diminished since 2019 (3.5% and 10.5% in 2019 to 0.0% and 1.1% in 2022, respectively). Moreover, the strains accumulating the most different antimicrobial resistances were those isolated from veal calves, with a maximum of 6 different resistances observed in *E. faecalis* and *E. faecium*. Conversely, a certain percentage of the strains showed no resistance, mainly those isolated from breeding hens, laying hens and pigs (33.3%, 46.0% and 33.3% of *E. faecalis* and 11.8, 40.4% and 12.4% of *E. faecium*, respectively).

In 2022, an NGS investigation of 19 enterococci was carried out based on the observed phenotypic antimicrobial resistance, with a focus on linezolid and tigecycline 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. However, cgMLST analyses of some strains sharing the same STs revealed close genetic relatedness between strains carrying linezolid resistance genes, among others.
Sequencing also revealed the presence of resistance genes explaining the resistant phenotype in 93.3% (n=83/89) of the individual phenotypes observed and allowed to genetically characterize resistances of human interest, such as resistance to critical antibiotics. Linezolid resistance in 2022 was characterized by the presence of transferable genes, as already observed in 2019 (Timmermans *et al.*, 2022) and in 2021 (see FASFC report). Except one, all the resistant strains analyzed (n=18) carried at least one gene, namely *optrA* (n=16 with 12 isolated from veal calves, 3 from pigs and 1 from breeding hens) or the combination of *optrA*/*poxtA* (n=1 isolated from veal calves).

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). Also, sequencing of enterococci revealed, for the first time to our knowledge, the presence of the *clpL* gene in 3 *E. faecalis* isolated from veal calves. This gene is known to be involved in many mechanisms such as resistance to stress, disinfectants or antibiotics (Jana *et al*., 2021; Pöntinen *et al*., 2017).
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 0.5 mg/L, as observed in 4 *E. faecalis* isolated from turkeys in 2022.

Virulence, a common trait of *Enterococcus* spp. was characterized by NGS. Different virulence factors have been identified within enterococci, ensuring different functions (adhesion, conjugation, biofilm formation, cytolytic activity, colonization) and allowing them to adapt to their environment. In 2022, 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 2022. 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 2022 confirmed these rates of resistances in *C. coli*. However, the epidemiological cut-off values recommended by EFSA for the analysis of ertapenem resistance are still under discussion.
Very high rates of resistance to ciprofloxacin and tetracycline were also detected in 2022 in both *C. coli* and *C. jejuni.* In *C. coli* isolated from samples of caeca from bovine animals very high rates of resistance to erythromycin were also detected whereas moderate resistance was observed in *C. jejuni*.
Combined resistance to both ciprofloxacin and erythromycin, which are considered critically important for treatment of campylobacteriosis, was moderate in *C. jejuni* from poultry, pigs and bovines.

Salmonella spp. isolated from food and feed samples, from pigs and bovine animals caecal content as well as from broilers and laying hens environmental samples were tested for antimicrobial susceptibility in 2022. 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 was the most prevalent and showed resistances mostly to ampicillin, sulfamethoxazole, and trimethoprim. In *Salmonella* spp. isolated from food and from broilers environmental samples however, the most prevalent serotype was Infantis that showed extremely high resistance level to (fluoro)quinolones, tetracycline and sulfamethoxazole and very high resistance levels to ampicillin. Overall, resistance to 3rd generation cephalosporins was rare in all categories of food-producing animals, food and feed, only one isolate recovered from veal calves displayed an ESBL phenotype. Resistance to colistin was very low as well, only detected in *S*. Paratyphi B Var. L(+) Tartrate +, *S*. Infantis isolated from broilers before slaughter, *S.* Dublin isolated from fresh poultry meat and in *S*. Senftenberg isolated from dog treats.

The monitoring of antimicrobial resistance in commensal indicator *E.coli* isolated from caecal samples of broilers, turkeys, fattening pigs and bovine animals taken at the slaughterhouse as well as from fecal samples of bovine animals, laying hens and breeding hens taken at farm level was carried out in 2022. Results of antimicrobial susceptibility testing show that the highest prevalences of resistances to (fluoro)quinolones, ampicillin, gentamicin, sulfamethoxazole and trimethoprim are found in *E. coli* isolated from broilers caecal samples. Resistances to 3rd generation cephalosporins were also the highest in *E.coli* isolated from turkeys and broilers caecal samples but were lower than in previous years. In *E. coli* isolated from pigs caecal content, even though the levels of resistance are overall lower than in other food-producing animals caecal content, an overall increase in resistance was observed in 2022 and a significant increase in resistance to ampicillin was confirmed in comparison to 2021. In fecal samples from animals taken at farm level, *E. coli* isolates showed overall lower resistance rates than in caecal samples. Still, an increase in resistance to several antimicrobials was noted in 2022 in comparison with previous years. Resistances to 3rd generation cephalosporins, gentamicin colistin and tigecycline were also higher in isolates from fecal samples of bovine animals than in those of laying hens and breeding hens. Meropenem was the only antimicrobial to which no resistance was detected in 2022 in any food-producing animal category.

A specific monitoring of ESBL, AmpC and/or carbapenemase producing *E. coli* was conducted in broilers, fattening pigs and bovine animals caecal samples and in fresh meat from these animals categories as well as from turkeys. This specific monitoring was also carried out in cows' raw milk and

in vegetables. No carbapenemase producing isolate was detected in 2022. In caecal sample from broilers a significant increase in resistance to chloramphenicol was noticed as well as an increase in resistance to most antimicrobials, especially (fluoro)quinolones. Although no significant difference in resistance rates was observed in comparison to 2021 in isolates retrieved from fattening pigs and bovine animals caeca, an overall increase in resistance to several antimicrobials is also noted in 2022. In fresh meat from broilers and turkeys and in raw milk, the prevalence remains stable, in fresh meat from fattening pigs and bovine animals and in vegetables, the prevalence of ESBL producing *E. coli* remains very low in 2022. A significant increase in resistance to ciprofloxacin was detected in 2022 in ESBL *E. coli* isolated from fresh meat of broilers. In food matrices an overall increase in resistances was also observed in 2022 in comparison with 2021.

The presence of MRSA in food-producing animals and their carriage of several AMR and virulence genes represents a public health risk. The extremely high prevalence (87.9%) observed in fattening pigs in 2022 with the new isolation method, is therefore a matter of concern.
Among the 225 MRSA isolated during the monitoring of 2022 in Belgium, 170 isolates (n=72 sows, n=98 fattening pigs isolates) were analysed by whole genome sequencing. All but one isolate were genotyped as LA-MRSA according to their STs/spa-types combinations. A clonal complex has not yet been assigned to the latter. However, this isolate is likely to belong to LA-MRSA according to our investigation. All MRSA isolated in 2022 were harboring the *mecA* gene and at least one tetracycline resistance genes, which are also characteristic of LA-MRSA. Several other resistance genes were observed and detailed in the results. Of particular concern is the carriage of the *cfr* gene, encoding a.o. resistance to the critically important antibiotic linezolid, observed again in 2022 in 3 isolates (n=1 sows and n=2 fattening pigs).
In addition to AMR genes, *qac* (precisely, *qacG* and *qacJ*) genes mediating resistance to quaternary ammonium compounds were observed in some MRSA isolates in 2022.
Moreover, several virulence genes associated with the immune evasion cluster (*sak, scn*), associated with toxins (*hlgA, hlgB, hlgC, seb* and *selw*) and exoenzymes (*aur*) were detected among the 170 MRSA isolates analysed in 2022. One sow isolate belonging to the ST398-t034 LA-MRSA type 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 carried the *tet(M)* gene, which is also typical of LA-MRSA and did not carry critically antimicrobial resistance gene (no *cfr* gene), neither qac disinfectant resistance gene. Altogether, this isolate would probably not have been recently transmitted from humans to sows, given the livestock-associated genetic background observed. The genes associated with the immune evasion cluster were not observed in the other isolates. The detection of the seb gene encoding an exotoxin known to be the source for multiple pathologies in humans in an isolate from fattening pigs highlights the importance of monitoring these different virulence factors in the future.
Several changes in the methodology used for the monitoring of MRSA have been made in 2022, including a new isolation method and the study of AMR through NGS rather than phenotypic susceptibility testings. The 2022 data will now serve as a new baseline for analyzing future trends in the prevalence of MRSA and the AMR genes they carry.

In 2022, 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 (85.6%), laying hens (69.9%), bovines (64.0%) and pigs (66.4%). Conversely, *E. faecalis* was isolated more frequently than *E. faecium* in broiler (65.4%) and turkey (92.0%) 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. Indeed, despite a high remaining rate, a significant decrease in the chloramphenicol resistance rate was observed in *E. faecalis* isolated from veal calves (-18.0%) in 2022 compared to 2019. In *E. faecium* isolated from broilers, a significant decrease (-17.7%) in ampicillin resistance was also observed in 2022

vs. 2019. 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. Linezolid-resistant strains were also observed in 2022, namely 13 *E. faecalis* isolated from veal calves (n=11) and pigs (n=2) and 5 *E. faecium* isolated from pigs (n=2), veal calves (n=2) and breeders (n=1). Additionally, 2 *E. faecium* isolated from veal calves (n=1) and breeders (n=1) were resistant to tigecycline, also the first time this resistance has been observed since 2019. No resistance to teicoplanin, or vancomycin was observed in 2022. In addition, a very low rate of daptomycin has been observed in *E. faecium* isolated from turkeys in 2022. This resistance was absent from all remaining animal categories, what could be related to the modification of the resistance threshold (from 4 to 8 mg/L) in 2021.
In general, a greater number of multidrug-resistant strains was observed in veal calves (60.2% of *E. faecalis)* and in broilers (60.3% of *E. faecium*). Moreover, the strains accumulating the most different antimicrobial resistances were those isolated from veal calves, with a maximum of 6 different resistances observed in *E. faecalis* and *E. faecium*.

A NGS investigation of 19 enterococci resistant to linezolid and/or tigecycline 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 some linezolid resistant strains was also observed. 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 93.3% (n=83/89) 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.

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Annex Ia Ib Ic NGS MRSA 2022

8. Abbreviations

AmpC : AmpC-type cephalosporins
AMR : Antimicrobial Resistance
CA-MRSA : community-associated MRSA
cgMLST : core-genome MLST
ESBL : Extended Spectrum Beta-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

9. References

Jennifer K. Bender, Vincent Cattoir, Kristin Hegstad, Ewa Sadowy, Teresa M. Coque, Henrik Westh, Anette M. Hammerum, Kirsten Schaffer, Karen Burns, Stephen Murchan, Carla Novais, Ana R. Freitas, Luísa Peixe, Maria Del Grosso, Annalisa Pantosti, Guido Werner. Update on prevalence and mechanisms of resistance to linezolid, tigecycline and daptomycin in enterococci in Europe: Towards a common nomenclature. Drug Resistance Updates 40 (2018) 25–39.

Bogaerts B, Nouws S, Verhaegen B *et al*. Validation strategy of a bioinformatics whole genome sequencing workflow for Shiga toxin-producing Escherichia coli using a reference collection extensively characterized with conventional methods. Microb Genom2021; 7:mgen000531.

Bystroń J, Podkowik M, Bania J, Krupa P, Schubert J. Distribution of enterotoxin genes in Staphylococcus aureus isolates from pork and pigs. *Medycyna Weterynaryjna*. 2015;71:341-344.

CLSI. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. 11th ed. CLSI standard M07. Wayne, PA : Clinical and Laboratory Standards Institute ; 2018.

Decision 2020/1729/EU on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria. Official Journal of the European Union 19.11.2020

Dubin, G. (2002). Extracellular Proteases of Staphylococcus spp. Biological Chemistry, 383(7-8). doi:10.1515/bc.2002.116
10.1515/bc.2002.116

European Food Safety Authority; Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in *Salmonella*, *Campylobacter* and indicator *Escherichia coli* and *Enterococcus* spp. Bacteria transmitted through food. EFSA Journal 2012; 10(6):2742. [64 pp.] doi:10.2903/j.efsa.2012.2742. Available online: www.efsa.europa.eu/efsajournal

European Food Safety Authority; Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in methicillin-resistant *Staphylococcus aureus* in food-producing animals and food. EFSA Journal 2012; 10(10):2897. [56 pp.] doi:10.2903/j.efsa.2012.2897. Available online: www.efsa.europa.eu/efsajournal

European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2019–2020. EFSA Journal 2022;20(3):7209. DOI : 10.2903/j.efsa.2022.7209. Available online: www.efsa.europa.eu/efsajournal

European Medicines Agency (EMA): Categorisation of antibiotics in the European Union - Answer to the request from the European Commission for updating the scientific advice on the impact on public health and animal health of the use of antibiotics in animals. 2020. https://www.ema.europa.eu/en/documents/report/categorisation-antibiotics-european-union-answer-request-european-commission-updating-scientific_en.pdf.

Farrera GuadalupePatriciaMací, Montes de Oca Jiménez R, Varela Guerrero J, Tenorio Borroto E, Rivera Ramírez F, Monroy Salazar HG, Yong Angel G, Salem AZM, Antibiotics susceptibility of quinolones against Salmonella spp. strains isolated and molecular sequenced from pigs: Target gyrA gene detection, Microbial Pathogenesis (2018), doi: 10.1016/j.micpath.2017.11.067.

Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol. 2004 Mar;186(5):1518-30. doi: 10.1128/JB.186.5.1518-1530.2004

S. Fiedler, J. K. Bender, I. Klare, S. Halbedel, E. Grohmann, U. Szewzyk and G. Werner. Tigecycline resistance in clinical isolates of Enterococcus faecium is mediated by an upregulation of plasmid-encoded tetracycline determinants tet(L) and tet(M). J Antimicrob Chemother 2016; 71: 871–881.

Frye, J. G., & Jackson, C. R. (2013). Genetic mechanisms of antimicrobial resistance identified in Salmonella enterica, Escherichia coli, and enteroccocus spp. isolated from U.S. food animals. Frontiers in Microbiology, 4, 135.

Ivbule Meldra. Occurrence of methicillin-resistant Staphylococcus aureus in pig industry. Summary of the doctoral thesis for the scientific degree of Dr.med.vet. Latvijas Lauksaimniecības universitāte. 2019.

Jana B et Biswas I. Significance of individual domains of ClpL: a novel chaperone from Streptococcus mutans. Biochemistry. PMC 2021 September 15.

Jiménez et al.: Antibiotic resistance, virulence determinants and production of biogenic amines among enterococci from ovine, feline, canine, porcine and human milk. BMC Microbiology 2013 13:288.

Kowalewicz, C., Timmermans, M., Fretin, D., Wattiau, P., & Boland, C. (2022). An in-house 45-plex array for the detection of antimicrobial resistance genes in Gram-positive bacteria. MicrobiologyOpen, e1341.

Larsen J, Sunde M, Islam MZ, Urdahl AM, Barstad AS, Larsen AR, Grøntvedt CA, Angen Ø. Evaluation of a widely used culture-based method for detection of livestock-associated meticillin-resistant Staphylococcus aureus (MRSA), Denmark and Norway, 2014 to 2016. Euro Surveill. 2017; 22(28):pii=30573.

Lopez M, Tenorio C, Del Campo R, Zarazaga M et Torres C. Characterization of the mechanisms of fluoroquinolone Resistance in Vancomycin-Resistant enterococci of Different Origins. Journal of Chemotherapy, Vol. 23 - n. 2 (87-91) – 2011.

Mannu L, Paba A, Daga E, Comunian R, Zanetti S, Duprè I et Sechi L A. Comparison of the incidence of virulence determinants and antibiotic resistance between Enterococcus faecium strains of dairy, animal and clinical origin. International Journal of Food Microbiology 88 (2003) 291– 304.

Pöntinen A, Aalto-Araneda M, Lindström M, Korkeala H. 2017. Heat resistance mediated by pLM58 plasmid-borne ClpL in Listeria monocytogenes. mSphere 2:e00364-17.

Rathnayake I U, Hargreaves M, Huygens F. Antibiotic resistance and virulence traits in clinical and environmental Enterococcus faecalis and Enterococcus faecium isolates Systematic and Applied Microbiology 35 (2012) 326– 333.

Schwarz S, Shen J, Kadlec K, Wang Y, Brenner Michael G, Feßler A T et Vester B. Lincosamides, Streptogramins, Phenicols, and Pleuromutilins: Mode of Action and Mechanisms of Resistance. Cold Spring Harb Perspect Med 2016;6:a027037.

Selleck E M, Van Tyne T et Gilmore M S. Pathogenicity of Enterococci. Microbiol Spectr. 2019 July ; 7(4).

Semedo T, Almeida Santos M, Silva Lopes M F, Figueiredo Marques J F, Barreto Crespo M T et Tenreiro R. Virulence Factors in Food, Clinical and Reference Enterococci: A Common Trait in the Genus? System. Appl. Microbiol. 26, 13–22 (2003).

Singh KV and Murray BE. Differences in the Enterococcus faecalis lsa Locus That Influence Susceptibility to Quinupristin-Dalfopristin and Clindamycin. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 2005, p. 32–39.

Smoglica, C.; Vergara, A.;Angelucci, S.; Festino, A.R.; Antonucci, A.; Marsilio, F.;Di Francesco, C.E. Evidence of Linezolid Resistance and Virulence Factors in Enterococcus spp. Isolates fromWild and Domestic Ruminants, Italy. Antibiotics 2022, 11, 223.

Soheili S, Ghafourian S, Sekawi Z, Neela V, Sadeghifard N, Ramli R et Hamat R A. Wide Distribution of Virulence Genes among Enterococcus faecium and Enterococcus faecalis Clinical Isolates. Hindawi Publishing Corporation The Scientific World Journal Volume 2014, Article ID 623174, 6 pages.

Timmermans M, Bogaerts B, Vanneste K, De Keersmaecker S C J, Roosens N H C, Kowalewicz C, Simon G, Argùdin M A, Deplano A, Hallin M, Wattiau1, Fretin D, Denis O et Boland C. Large diversity of linezolid-resistant isolates discovered in food-producing animals through linezolid selective monitoring in Belgium in 2019. J Antimicrob Chemother 2022; 77: 49–57.

Torres C, Alonso CA, Ruiz-Ripa L, León-Sampedro R, del Campo R, Coque TM. 2018. Antimicrobial resistance in Enterococcus spp. of animal origin. Microbiol Spectrum 6(4):ARBA-0032-2018.

Verreault D, Ennis J, Whaley K, Killeen SZ, Karauzum H, Aman MJ, Holtsberg R, Doyle-Meyers L, Didier PJ, Zeitlin L, Roy CJ. Effective Treatment of Staphylococcal Enterotoxin B Aerosol Intoxication in Rhesus Macaques by Using Two Parenterally Administered High-Affinity Monoclonal Antibodies. Antimicrob Agents Chemother. 2019 Apr 25;63(5):e02049-18. doi: 10.1128/AAC.02049-18.

Vrieling M, Tuffs SW, Yebra G, van Smoorenburg MY, Alves J, Pickering AC, Park JY, Park N, Heinrichs DE, Benedictus L, Connelley T, Seo KS, McCormick JK, Fitzgerald JR. 2020. Population Analysis of Staphylococcus aureus Reveals a Cryptic, Highly Prevalent Superantigen SElW That Contributes to the Pathogenesis of Bacteremia. mBio. 2020 Oct 27;11(5):e02082-20. doi: 10.1128/mBio.02082-20.

World Health Organization. 2019. Critically important antimicrobials for human medicine, 6th revision. Geneva. Licence: CC BY-NC-SA 3.0 IGO.

Zaheer R, Cook SR, Barbieri R, Goji N, Cameron A, Petkau A, Ortega Polo R, Tymensen L, Stamm C, Song J, Hannon S, Jones T, Church D, Booker CW, Amoako K, Van Domselaar G, Read RR & McAllister TA. Surveillance of Enterococcus spp. reveals distinct species and antimicrobial resistance diversity across a One-Health continuum. Scientific Reports | (2020) 10:3937.

10. Acknowledgments

This work was funded by the FAVV-AFSCA. We thank Mickael Cargnel for his help in the statistical analysis of our data. We also thank the Transversal Activities in Applied Genomics service of Sciensano for the sequencing and the development and maintenance of the Galaxy tools and pipelines used for NGS analyses.