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Impact of amoxicillin treatment on intestinal microbiota and antibiotic resistances in calves

Tony Rochegüe

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Tony Rochegüe. Impact of amoxicillin treatment on intestinal microbiota and antibiotic resistances in calves. Microbiology and Parasitology. Université de Lyon, 2021. English. NNT : 2021LYSE1321 . tel-03774899

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N°d'ordre NNT : 2021LYSE1321

THESE de DOCTORAT DE L'UNIVERSITE DE LYON

opérée au sein de
l'Université Claude Bernard Lyon 1

Ecole Doctorale ED 205
(Ecole Doctorale Interdisciplinaire Sciences Santé)

Spécialité de doctorat : Microbiologie

Soutenue publiquement le 14/12/2021, par :

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Acknowledgments

La première personne que je tiens à remercier est bien évidemment Agnese LUPO, qui a dirigé cette thèse (et a également encadré mon stage de M2 avant cela). Je ne saurais comment t'exprimer ma gratitude et ma reconnaissance pour tout ce que tu m'as apporté durant ces dernières années, qui ont été pour le moins riches en émotions et en rebondissements. Merci de m'avoir tant appris, tant partagé et de m'avoir encouragé à vivre cette aventure de laquelle je ressors grandi, professionnellement et personnellement.

J'adresse ensuite mes remerciements à Tristan FERRY pour avoir accepté de co-diriger cette thèse et pour ses conseils et encouragements lors de mes CST notamment.

Je souhaite également remercier l'ensemble des membres du jury : merci à Delphine BIBBAL, Benoît DOUBLET et Alain HARTMANN d'avoir accepté d'être rapporteurs de cette thèse, à Anne VIANNEY en tant qu'examinatrice et à Zorée DJELOUADJI et Thomas JOVÉ pour leur participation. Merci à chacun d'entre vous pour l'intérêt porté à mon travail.

Je suis aussi reconnaissant envers Jean-Yves MADEC, chef de l'équipe AVB, et Marisa HAENNI, chef de l'unité adjointe, pour leur confiance et leur accueil au sein de l'équipe. Jean-Yves, je vous remercie sincèrement de m'avoir accompagné avec sollicitude durant le premier confinement. Marisa, je te remercie pour ta présence à tout instant et ton aide sur de nombreux aspects.

Je pense aussi évidemment à l'ensemble de l'équipe AVB grâce à laquelle j'ai rencontré des personnes formidables et avec qui j'ai eu un réel plaisir de travailler. Que ce soit directement ou indirectement, chacun de vous a apporté sa pierre à l'édifice et je vous en suis très reconnaissant. Merci en particulier à Henok, Hiba, Raquel, Rodolphe et Youssef pour tous ces moments partagés autour d'un verre ou d'un Mölkky.

J'étends mes remerciements à toutes les personnes avec lesquelles j'ai collaboré au cours de ces dernières années : Hervé BLOTTIERE et Stanislas MONDOT pour nos échanges et vos précieux conseils durant les CST ; Sandrine HUGHES et Benjamin GILLET pour nos discussions enrichissantes autour du SpeedVac ; Claire HOEDE et Joanna FOURQUET pour votre contribution sur la partie de métagénomique et votre accueil lors de mon déplacement à Toulouse ; Chloé ASTRUC pour m'avoir mis en lien avec l'ensemble des éleveurs, et je remercie par ailleurs chacun d'entre eux pour m'avoir permis de réaliser l'échantillonnage des veaux de leur ferme.

Sur un aspect plus personnel, je tiens tout d'abord à remercier mes amis, avec qui je passe de grands moments de joie depuis des années et qui m'ont apporté beaucoup de réconfort sur les dernières. Je pense tout particulièrement à toi Max (d'ailleurs tu aurais peut-être dû être mentionné dans le paragraphe « famille » tant ce que nous avons partagé depuis 2015 est intense, sincère, profond, précieux), tu sais déjà tout mais encore une fois : merci. Je pense aussi évidemment à Valentin, mon ami depuis tant d'années : tu es un pilier pour moi mon Dodet, merci d'être là, merci d'être toi, merci d'emménager à 3 pas de chez moi, je suis ravi de cette perspective de passer encore plus de moments avec toi. Et toi Ambre, que dire ! J'aime ta folie, ton enthousiasme, ton naturel, mais surtout je te remercie d'être une amie sur laquelle je peux compter de jour comme de nuit. Je pense aussi très fort à vous Bastien et Emma, nous avons déjà partagé tant de moments ensemble depuis toutes ces années d'amitié, je ne compte plus les fous-rires, les chansons, les jeux, les bringues, et j'attends les prochaines avec une grande impatience, vous êtes tous les deux si importants pour moi. Merci également à l'équipe de Lyon, toujours fidèle, avec qui c'est à chaque fois un régal de passer du temps : Cadiou, Céline, Michaël, Nicolas et en particulier toi Rémi, qui m'a tant fait rire durant ces dernières années et qui m'a permis de penser à autre chose sur ce fameux toit de l'hôpital de Verdansk.

Et Amandine, toi qui réussis à me faire sourire quand ça ne va pas, à me remonter le moral dans les moments les plus durs. Ton soutien sans faille m'a permis de donner le meilleur de moi-même durant ces dernières années. Merci d'illuminer ma vie et me rendre plus heureux chaque jour.

Je tiens aussi bien évidemment à remercier ma famille, à commencer par mes parents et ma sœur, je vous suis infiniment reconnaissant pour tout ce que vous fait pour moi, merci pour votre soutien et votre bienveillance sans faille, je vous aime tant tous les trois ! Un grand merci ensuite à ma grand-mère Jeannette, pour sa profonde gentillesse et tous ses bons petits plats qui nous permettent de partager des moments agréables en famille. J'ai également une pensée particulière pour mon grand-père, Paul, qui nous a quitté en janvier dernier : à toi qui avait un esprit si curieux et fin, je dédie ce travail.

Abbreviation list

AAC	Aminoglycoside acetyltransferase
APH	Aminoglycoside phosphotransferase
AMR	Antimicrobial resistance
ANT	Aminoglycoside nucleotidyltransferase
ARG	Antibiotic resistance genes
ESBL	Extended-spectrum beta-lactamases
GIT	Gastrointestinal tract
HMO	Human milk oligosaccharides
IBD	Inflammatory bowel disease
IHMS	International Human Microbiome Standards
IM	Intestinal microbiota
LPS	Lipopolysaccharide
MetaHIT	Metagenomics of the Human intestinal tract
MGE	Mobile genetic elements
pAmpC	Plasmid-encoded AmpC
PBP	Penicillin-binding proteins
qPCR	Quantitative polymerase chain reaction
QRDR	Quinolones resistance determining region
SCFA	Short-chain fatty acids
TCS	Two-component systems
TLR	Toll-like receptors

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Résumé

L'utilisation inadéquate d'antibiotiques chez l'homme et l'animal a accéléré l'émergence et la dissémination de bactéries résistantes aux antibiotiques qui, constituent une menace pour la santé mondiale. Les bactéries porteuses de gènes conférant une résistance aux antibiotiques (ARGs) sont souvent retrouvées au sein du microbiote intestinal (MI) chez les mammifères dès la naissance et font partie intégrante du résistome. Les veaux sont particulièrement préoccupants au sujet du portage des ARGs au sein du MI. Le résistome des veaux est acquis via de multiples sources telles que la mère, le colostrum, la nourriture ou plus généralement l'environnement de l'élevage.

Les antibiotiques sont considérés comme des perturbateurs majeurs du MI, notamment en impactant les espèces bactériennes bénéfiques principalement appartenant au phylum des Firmicutes mais également en favorisant l'émergence de pathogènes potentiels appartenant souvent au phylum des Protéobactéries. La pression de sélection faite par l'antibiotique joue un rôle majeur dans le développement de bactéries résistantes au sein de l'intestin, ce qui augmente le potentiel de dissémination à d'autres hôtes (humains ou animaux) par voie fécal-oral ou à travers la chaîne alimentaire.

L'amoxicilline est l'un des antibiotiques les plus utilisés dans le monde que ce soit dans la médecine humaine ou vétérinaire. De plus, en médecine vétérinaire, il est recommandé d'utiliser l'amoxicilline avec prudence, mais, en traitement de première intention quand cela est possible. L'effet de cet antibiotique a été étudié dans le MI de plusieurs hôtes mettant en évidence l'augmentation de ARGs, conférant ainsi une résistance à l'amoxicilline et affectant la composition du MI.

Malgré l'usage prédominant et le rôle important de l'amoxicilline en médecine vétérinaire, les études longitudinales analysant l'effet du traitement à base d'amoxicilline sur le MI chez le veau manquent. Pour combler ce manque de connaissance sur le sujet, l'effet de l'amoxicilline a été analysé dans un groupe de veaux (n = 17) hébergés dans des élevages commerciaux et atteints d'omphalite via trois axes expérimentaux.

Dans un premier temps, des tests de qPCR ciblant les principaux ARGs retrouvés chez les Entérobacterales ainsi que les intégrases codant pour les gènes propres aux intégrons (intI1/2), qui sont considérés comme un indice de développement de résistance multiples, ont été désignées et validées. Ces tests ont servi à quantifier les ARGs contenus dans les fèces des veaux récoltés avant, à la fin et une semaine après le traitement à base d'amoxicilline. La

quantité du gène *bla*_{TEM}, responsable de la résistance à l'amoxicilline, a augmenté à la fin du traitement et a diminué une semaine après l'arrêt de la thérapie chez les veaux traités. Dans le groupe contrôle constitué de cinq veaux non traités, *bla*_{TEM} a diminué tout au long de l'analyse. D'autres ARGs ont également eu la même dynamique que *bla*_{TEM}, tels que *tetA*, conférant résistance à la tétracycline, *strA/B* conférant résistance à la streptomycine, *aph(3')*-*Ia* conférant résistance à la kanamycine, et *intI1* codant pour l'intégrase des intégrons de classe 1. Ces résultats suggèrent une co-sélection potentielle de l'amoxicilline à des résistances à d'autres classes d'antibiotiques.

La quantité de bactéries résistantes a également été évaluée par culture sur des milieux de croissance sélectifs pour les bactéries Gram-négatives, contenant ou non de l'amoxicilline à une concentration capable de sélectionner des *Escherichia coli* résistants. Le nombre d'unités formant colonies (CFUs) obtenues ne différait pas significativement entre le milieu contenant de l'amoxicilline et sans. L'absence de différence statistiquement significative pourrait être due à la variation de la quantité de CFUs dans l'IM des veaux, même avant le traitement à l'amoxicilline. Une telle variation pourrait être attribué à l'âge différent des veaux, qui affecte profondément la composition de la MI et le contenu des ARG. Les isolats d'*E. coli* obtenus à partir du dénombrement des CFUs ont été analysés plus en détail. En particulier, à partir de la gélose utilisée pour le dénombrement des CFUs, toutes les colonies présentant une morphologie unique ont été purifiées et l'ADN extrait. Le génome des isolats (n = 152) a été séquencé générant de courtes lectures par une approche Illumina. L'analyse bioinformatique a permis de déterminer le phylogroupe des isolats. Le phylogroupe A était le plus représenté (n = 57/152), suivi du phylogroupe B1 (n = 54/152), du phylogroupe D (n = 38/152) et B2 (n = 3/152). Les types de séquences (ST) les plus fréquents appartenaient au CC10 (ST10/34/48/167), suivi du CC23 (ST23 et ST88) et CC155 (ST56 et ST58). Le gène *bla*_{TEM1A/B} était présent dans toutes les souches de *E. coli* résistantes à l'amoxicilline (n = 114/152). Ce gène était souvent associé à l'IS26 et l'IS91 mais également retrouvé à proximité de l'opéron *mer*. Le gène *bla*_{TEM-1} a également été retrouvé à proximité de gènes de résistance aux aminosides et à la tétracycline. Des intégrons de classe 1 ont été retrouvés dans la plupart des isolats sous la forme de dix différents variants. Les plasmides les plus fréquemment retrouvés dans les isolats sont du type IncFII, IncFIA-IB et IncQ1. Des isolats génétiquement apparentés ont été mis en évidence chez des veaux résidant dans différentes fermes, la similarité augmentant avec la diminution de la distance géographique entre les fermes. Les clones d'*E. coli* ont pu coloniser différents veaux résidant dans la ferme non simultanément, suggérant un réservoir environnemental ou un vecteur vivant où de tels clones pourraient persister dans la même ferme.

Les résultats préliminaires de l'analyse de l'ADN extrait des selles par séquençage shotgun, ont suggéré une diminution, à la fin du traitement, des phylums Firmicutes et Verrucomicrobia, alors que ceux des Bacteroidetes, Actinobactéries et Protéobactéries augmentaient. Une semaine après la fin du traitement, les Actinobactéries ont diminué et une légère augmentation des Firmicutes s'est produite, tandis que les Verrucomicrobia ne sont pas revenus à leur état d'avant traitement.

En intégrant plusieurs axes méthodologiques, notre étude ouvre la voie à la compréhension du rôle de l'amoxicilline dans la composition et les résistances contenues dans le MI des veaux. Ces approches combinées sont nécessaires pour comprendre l'effet global de l'antibiothérapie pour améliorer l'utilisation des antibiotiques et trouver des stratégies pour limiter les conséquences néfastes des antibiothérapies sur la santé de l'hôte et pour contrer la sélection des ARGs dans l'intestin.

Keywords : *E. coli*, *bla_{TEM-1}*, qPCR, *intI1*, *tetA*, *strA*, *strB*, *aph(3')-Ia* , IncFII, IncQ1, shotgun, injection intra-musculaire.

Abstract

Antibiotics misuse in humans and animals accelerated global emergence and dissemination of antimicrobial resistance, which nowadays constitutes a threat to global health. Bacteria harboring antibiotic resistance genes (ARGs) are known to be resident in the intestinal microbiota (IM) of mammals since birth, constituting the so-called resistome. Calves represent a special concern because of the high prevalence of ARGs within their IM. The calves resistome is acquired from multiple sources including the mother, colostrum, food, or more in general the farm environment.

Antibiotics are major disruptors of the IM, depleting bacterial species beneficial for the host health, mostly belonging to the Firmicutes phylum, and favoring the emergence of potential pathogens commonly belonging to the Proteobacteria phylum. The antibiotic pressure plays a major role on the selection of resistant bacteria within the gut, which in turn can potentially contaminate the environment, increasing the risk of dissemination to other hosts (humans or animals) through the fecal-oral route or the food chain. Amoxicillin is one of the most used antibiotics worldwide in human and veterinary medicine. In veterinary medicine, amoxicillin is also considered to be used with prudence, but, when possible, as first-line treatment. The effect of this antibiotic has been investigated in the IM of multiple hosts, highlighting an increase of ARGs conferring resistance to amoxicillin and perturbation of the IM composition.

Despite the large usage and the important role of amoxicillin in veterinary medicine, longitudinal studies analyzing the effect of amoxicillin treatment on the IM of calves are lacking. To fill in this gap of knowledge, the effect of amoxicillin treatment was analyzed in a group of calves hosted in commercial farms and suffering from omphalitis via three experimental axes.

First, qPCR assays targeting the major ARGs found in Enterobacterales and also integrases encoding genes proper to integrons (*intI1/2*), whose presence is index of multidrug resistance, were designed and validated. These assays were used to quantify ARGs in stools of calves (n = 17) collected before amoxicillin treatment, at the end of the amoxicillin therapy, and one week after the end of the therapy. The amount of the *bla*_{TEM} gene, responsible for amoxicillin resistance, increased after amoxicillin treatment and decreased after therapy withdrawal in treated calves, whereas in the control group, constituted by untreated calves

(n=5) that were sampled at the same pace of the treated ones, *bla*_{TEM} constantly decreased. Other genes followed the same dynamic as *bla*_{TEM}, such as *tetA*, conferring resistance to tetracycline, *strA/B* conferring resistance to streptomycin, *aph(3')-Ia* conferring resistance to kanamycin, and *intI1* encoding the integrase of class 1 integrons. This suggests a potential co-selection by amoxicillin of resistance to other classes of antibiotics.

Amount of resistant bacteria was also evaluated by cultivation on growth media selective for Gram-negative bacteria, amended or not with amoxicillin at a concentration capable to select resistant *Escherichia coli*. The count of the obtained colony forming units (CFUs) did not significantly differed between the medium containing selective amoxicillin and the free one. The lack of statistically significant difference could be due to the variation in CFUs amount in the calves' IM, even before amoxicillin treatment. In turn, such variation could be attributable to the different age of the calves, which deeply affects IM composition and ARGs content. The *E. coli* isolates obtained from the CFUs enumeration were analyzed further. In particular, from the plate used for the CFUs enumeration, all the colonies displaying different morphology from each other were purified and DNA extracted. The genome of the isolates (n = 152) was sequenced generating short reads by an Illumina approach. Bioinformatic analysis allowed to determine the phylogroup of the isolates. The phylogroup A was the most represented (n = 57/152), followed by phylogroup B1 (n = 54/152), phylogroup D (n = 38/152), and B2 (n = 3/152). The most frequent sequence types (STs) belonged to CC10 (ST10/34/48/167), followed by CC23 (ST23 and ST88), and CC155 (ST56 and ST58). The *bla*_{TEM-1A/B} gene was present in all amoxicillin-resistant *E. coli* (n = 114/152). This gene was often associated to IS26 and IS91 and located close to a *mer* operon. The *bla*_{TEM-1} gene was also found close to genes conferring aminoglycosides and tetracycline resistance. Class 1 integrons were found in most of the isolates and ten variants were detected. The most represented plasmids in the isolates were of type IncFII, IncFIA-IB, and IncQ1. Genetically related isolates occurred in calves residing in different farms, with similarity increasing with decreasing of the geographical distance between farms. *E. coli* clones were able to colonize different calves residing in the farm not contemporaneously, suggesting an environmental reservoir or living vector where such clones could persist in the same farm.

Preliminary results from the analysis of DNA extracted from stools by shotgun sequencing, suggested a decrease, at the end of the treatment, of the Firmicutes and Verrucomicrobia phyla, whereas Bacteroidetes, Actinobacteria and Proteobacteria relative abundance increased. One week after the end of the treatment, Actinobacteria decreased and a

slight increase of Firmicutes occurred, whereas Verrucomicrobia did not return to the baseline.

Integrating multiple methodological axes, our study paved the way to understand the role of amoxicillin therapy in the IM composition and resistances of calves. These combined approaches are necessary to understand the global effect of antibiotic therapy for improving antibiotic usage and find strategies to limit the negative consequences of antibiotic therapies on the host health and to counteract the selection of ARGs in the gut.

Keywords : *E. coli*, *bla*_{TEM-1}, qPCR, shotgun, *intI1*, intra-muscular injection, IncFII, IncQ1, *tetA*, *strA*, *strB*, *aph(3')-Ia*



I. Introduction

The globalization of antibiotic use and misuse in humans and as growth promoters for food-producing animals accelerated antimicrobial resistance (AMR) emergence and propagation^{1,2}. Thus, AMR is a global health issue of increasing magnitude³, concerning not only the traditionally appointed human clinical settings but also non-clinical environments, which are becoming increasingly recognized as an important factor in the evolution and dissemination of AMR.

Commensal, pathogenic, and environmental microorganisms contribute to the reservoir of antibiotic resistance genes (ARGs) collectively forming the antibiotic resistome⁴, which is present in the intestinal microbiota (IM) of mammals since birth⁵⁻⁸. Newborn animals can acquire AMR from their mothers during delivery, from colostrum or other food sources⁹, or the environment, including contamination from nursing personnel, farmworkers and feeding equipment¹⁰⁻¹³.

In IM, antibiotic therapies are disruptors of the bacterial components of the IM killing species beneficial to the host health, for example, species of the Firmicutes phylum, and favoring the emergence of potential pathogens, most often belonging to the Proteobacteria phylum. Among these, AMR of clinical importance occurs at high frequency. Furthermore, antibiotic therapies select resistant bacteria in the IM that once excreted in the environment can propagate to further hosts. This is a particular issue in the farm environment, where AMR can be transmitted from livestock to humans through the fecal-oral route, by the food chain contamination and through the environment^{10,14-17}.

In the panorama of food-producing animals, young bovines constitute a special concern for the high prevalence of AMR. According to the 2019 report of the RESAPATH, a surveillance system based on susceptibility data provided by a network of diagnostic laboratories, in France, *Escherichia coli* was the most frequent species isolated from gastrointestinal pathologies, which in turn were the most common pathologies reported in calves. In this context, the occurrence of resistance to last-generation cephalosporins, a major resistance mechanism for human and veterinary medicine, decreased to 3% (n = 3871). However, resistance to amoxicillin occurred at high levels, 85%. High levels of resistance were reported for other antibiotic classes: 83% for streptomycin, 56% for neomycin, 77% for tetracycline, and 40% for sulfonamides-trimethoprim¹⁸. Similar resistance proportions were reported in *E. coli* from diseased calves in Germany^{19,20}.

The occurrence of infections is more common in young animals, thus frequent antibiotic therapies are necessary, a factor that could be in accordance with the high

occurrence of AMR in these hosts. Furthermore, the consequences of antibiotic therapies are more dramatic on IM in maturation, likewise IM of humans. However, not all antibiotic classes exert the same selective pressure on mammals' intestines.

In the following paragraph, notions on IM construction of mammals will be provided, along with current knowledge on antibiotics-IM interaction, and principal AMR against currently available antibiotics encountered in Enterobacterales. To this order, belong species such as *E. coli*, which harbor AMR mechanisms of peculiar concern for global health, and are major pathogens for humans and animals.

1. Antibiotics and antibiotic resistance mechanisms in Enterobacterales

The advent of antimicrobials during the 20th century has revolutionized human medicine by saving millions of lives and increasing the life expectancy of 15 years, on average²¹. This medical revolution was signed by the mass production of penicillin in the 1940s, protecting against bacterial endocarditis, meningitis, and pneumonia. More generally, the period from 1940 to 1960 was considered the golden age of antibiotic discovery²². This age ended with the emergence of hard-to-treat multidrug-resistant bacteria resulting in high morbidity and mortality rates of infectious diseases. Today, new antibiotics are limited in discovery, because of the costly investment that pharmaceutical development implies²³. Besides, AMR will fatally emerge and disseminate. In fact, ARGs have been found also in pristine sources not influenced by the presence of modern-day antibiotics like in a mummy of the 11th century AD, in 30000-year-old permafrost sediment, and in a cave in New Mexico that had been apart from humans for millions of years^{24,25}. The ubiquitous presence of ARGs resides in the fact that besides protecting bacteria from antibiotics, they have other physiological functions in the cell^{14,15,26,27}. Furthermore, the antibiotic resistome contains cryptic genes that may evolve into resistance genes or become functional under appropriate conditions^{4,28}. Commensal or environmental bacteria are often a source of this kind of genes, which can transfer to pathogenic microbes^{29,30}. An example is provided by the *qnrA* and *bla*_{CTX-M} determinants originated from non-pathogenic microbes that were transferred to Enterobacterales and afterward to other potential pathogens^{31,32}. The following subparagraphs will report the main antibiotics' mechanisms of action, relative resistances and genetic elements encountered in Enterobacterales.

1.1. Antibiotics targeting the cell wall synthesis, resistance mechanisms and genetic elements

Antibiotics targeting the cell wall, in Gram-negative bacteria, are beta-lactams and polymyxins. Beta-lactams impede peptidoglycan formation via the inhibition of penicillin-binding proteins (PBPs) inducing cell lysis³³. The major mechanism of beta-lactam resistance is based on the beta-lactam ring hydrolyzation by enzymes named beta-lactamases. These enzymes are grouped in four classes, A, B, C, and D, according to Ambler classification³⁴.

The *bla*_{TEM} gene is the most prevalent gene encoding for beta-lactamases, including more than 220 variants³⁵. The first reported variant of this gene, *bla*_{TEM-1}, can hydrolyze penicillins, aminopenicillins, and early cephalosporines. More recent variants, like the enzyme encoded by *bla*_{TEM-52}, have acquired an ESBL phenotype via amino acid substitutions (E104K, M182T, and G238S), allowing hydrolysis of third- and fourth-generation cephalosporines³⁶. Nowadays, *bla*_{TEM-1} and *bla*_{TEM-52} are the most prevalent *bla*_{TEM} beta-lactamases disseminated in Enterobacterales via mobile genetic elements. The *bla*_{TEM-1} gene is associated with the insertion sequence IS26 and located on Tn2 transposons, whereas *bla*_{TEM-52} is included on Tn3 transposons in turn located on IncI plasmids, found in isolates of human and animal origin^{37,38}.

As *bla*_{TEM}, *bla*_{SHV} beta-lactamases are composed of penicillinases and ESBL encoding variants. These ESBL variants, such as *bla*_{SHV-12}, acquired substitution (G238S) from *bla*_{SHV-1}³⁹. The *bla*_{SHV-12} gene is one of the most disseminated in multiple ecological niches via plasmids of the IncX3 and IncI1 type^{40,41}.

Class B beta-lactamases, or metallo beta-lactamase, were discovered in the 1990s. The most common disseminated genes of this group are *bla*_{VIM}, *bla*_{IMP} and *bla*_{NDM}, which confer resistance to all currently available beta-lactam antibiotics. These genes are commonly found in Enterobacterales but also in non-fermenters (*Pseudomonas aeruginosa* and *Acinetobacter baumannii*)⁴².

The *bla*_{VIM} gene has spread all over the world harbored on IncN and IncI1 plasmids⁴³. The *bla*_{IMP} gene has disseminated via IncL/M, IncA/C plasmids and integrated into class 1 integrons⁴⁴. The more recently discovered gene was *bla*_{NDM}, which has been found on several self-transferable plasmids belonging to IncA/C, IncF, IncL/M types favoring the dissemination of the gene⁴². Class C beta-lactamases located on plasmids (pAmpC) are increasing in prevalence with *bla*_{CMY} and *bla*_{DHA} as the most represented globally. The *bla*_{CMY}

gene comprises 155 variants, including *bla*_{CMY-2}, the most common pAmpC in humans and animals worldwide⁴⁵⁻⁴⁷. This gene is linked to the insertion sequence *ISEcp1*, which has mobilized *bla*_{CMY-2} on different plasmids belonging to the IncI1 and IncK groups⁴⁸.

The *bla*_{DHA} gene includes nine variants and among those *bla*_{DHA-1} is the most propagated. This group is the second most prevalent pAmpC in Enterobacterales. The *bla*_{DHA-1} gene is mobilized by the insertion sequence *ISCR1*, and it can be located on class1 integrons and plasmids of the IncI1 and IncF type^{49,50}.

The *bla*_{CTX-M} gene encodes for ESBL enzymes, the most disseminated worldwide. In this family of genes four groups, *bla*_{CTX-M-1}, *bla*_{CTX-M-2}, *bla*_{CTX-M-8}, and *bla*_{CTX-M-9}, are recognized based on amino acid sequence similarity of the corresponding enzymes⁵¹. The groups include, in turn, variants such as *bla*_{CTX-M-3}, *bla*_{CTX-M-15}, *bla*_{CTX-M-55} for the *bla*_{CTX-M-1} group, *bla*_{CTX-M-14}, *bla*_{CTX-M-27} for the *bla*_{CTX-M-9} group, which have disseminated worldwide with enormous success. In 2017, *bla*_{CTX-M-15} was the most widespread variant all over the world except in South America, where *bla*_{CTX-M-2} and *bla*_{CTX-M-8} are dominant, and in Asia, where *bla*_{CTX-M-14} is the most common⁵². The genes *bla*_{CTX-Ms} are associated with several insertion sequences, for instance, *ISEcp1*, *ISCR1*, *IS26*, and *IS10*, but also to Tn2 transposons and integrons, and of course plasmids of several types (IncF, IncHI1, IncHI2, IncI1, IncK, and IncN)⁵³.

In Enterobacterales, mostly the OXA-48 enzyme and its derivatives represent class D beta-lactamases. The *bla*_{OXA-48} gene was detected for the first time in Europe in 2001 and then in isolates from companion animals as part of the composite transposon Tn1999.2, in turn, located on an IncL plasmid^{54,55}. The most widespread derivative, *bla*_{OXA-181}, is associated with the *ISEcp1* insertion sequence, in turn, inserted in a Tn2013 transposon, located on an 84-kb mobile IncT-type plasmid⁵⁶. *bla*_{OXA-181} is widely disseminated among humans in Asia and Europe^{57,58}. Sporadically, the *bla*_{OXA-23} gene, which is principally detected in *A. baumannii*⁵⁹, is reported from Enterobacterales on IncF and IncK plasmids and also on Tn5393 transposon⁶⁰⁻⁶². Overall, beta-lactamases encoding genes have been reported worldwide in isolates from humans and animals⁶³⁻⁶⁸.

Polymyxins are small polypeptides that electrostatically bind to the lipopolysaccharide (LPS) resulting in the permeabilization of the outer membrane and loss of cell viability⁶⁹. Resistance to polymyxins is caused by mutations in the two-component systems (TCS) PhoP/PhoQ and PmrA/PmrB. These punctual mutations are resulting in constitutive activation and subsequent overexpression of LPS-modifying genes⁷⁰. In particular, the activation of the PmrA/PmrB TCS leads to the upregulation of the *pmrHFJKLM* and *ugd* operons that mediate

the synthesis of lipid A⁷¹. The other TCS PhoP/PhoQ is known to contribute to polymyxin resistance by indirectly activating the PmrA/PmrB TCS and inducing LPS modification⁷².

In November 2015, a plasmid-mediated gene conferring resistance to colistin, named *mcr-1*, was found in Enterobacterales⁷³. Since then, ten *mcr* gene variants have been reported⁷⁴. The *mcr-1* gene is worldwide disseminated among humans, animals, and environmental compartments in Enterobacterales and is often found on transposons flanked by two insertion sequences IS*Apl1* belonging to the IS30 family. IncI2 and IncX4 are the most dominant plasmid type carrying *mcr-1*^{73,75,76}. In terms of prevalence, the *mcr1* gene is followed by the *mcr-3* gene harbored on IncH2 plasmids^{77–79}.

1.2. Antibiotics targeting nucleic acid synthesis, resistance mechanisms and genetic elements

Quinolones inhibit DNA gyrases via modification of the replication fork leading to cell death⁸⁰. Resistance to quinolones is mediated by point mutations in GyrA and ParC enzymes concerning the QRDR (Quinolones Resistance Determining Region). The most common mutations found in *E. coli* are GyrA S83L and ParC S80I⁸¹. One mutation causes the reduction of the susceptibility of the bacteria to quinolones, whereas the accumulation of mutations increases the resistance levels⁸² and can affect also fluoroquinolones' actions^{83,84}.

Quinolones resistance in Enterobacterales is also based on acquired mechanisms mediated by plasmidic *qnr* genes, whose proteins bind to DNA gyrase and topoisomerase, or by modification of the antibiotic via acetylation mediated the acetylase encoded by the *aac(6')-Ib-cr* gene^{85,86}. Among the *qnr* genes, *qnrB* and *qnrS* are the most prevalent and often co-localized on the same plasmid with *bla*_{CTX-M} genes^{87–90}. The *aac(6')-Ib-cr* derives from the *aac(6')-Ib* gene, mediating aminoglycosides resistance, via two substitutions (W102R and D179Y)⁹¹. The *aac(6')-Ib-cr* gene is often located on self-mobilizable IncFII plasmids together with the *bla*_{CTX-M-15} gene in human and animal isolates^{68,92,93}.

Folic acids are precursors of nucleic acids and their metabolic pathway can be inhibited by two antibiotic families: the sulfonamides and the trimethoprim. Genes conferring sulfonamides resistance code alternative enzymes, which allow the metabolization of folic acids and consist of *sul1*, *sul2*, and *sul3*, which are commonly found in Gram-negative bacteria⁸². The *sul1* gene is part of the class1 integrons and is the most prevalent among *sul*

genes⁹⁴⁻⁹⁶. Each gene seems to be localized on a specific plasmid type (IncI1 for *sul1*, IncFII for *sul2*, and IncI1 for *sul3*)⁹⁶.

Trimethoprim resistance is also based on alternative enzymes, such as the dihydrofolate reductase A, encoded by *dfrA* genes among which three variants are common in Enterobacterales: *dfrA1*, *dfrA12*, and *dfrA17*. These genes are located on class 1 and 2 integrons in human and animal isolates^{97,98}.

1.3. Antibiotics targeting proteins synthesis, resistance mechanisms and genetic elements

The bacterial translation in Enterobacterales can be inhibited by multiple antibiotics like aminoglycosides, tetracyclines, and phenicols. These molecules binding to the 16S ribosomal RNA of the 30S subunit induce cell death, in the case of aminoglycosides, or inhibition of growth, in the case of tetracyclines and phenicols⁹⁹⁻¹⁰¹.

The aminoglycosides resistance is mostly based on the modification of the drug by aminoglycosides acetyltransferase (AAC), aminoglycosides phosphotransferase (APH), and aminoglycosides nucleotidyltransferase (ANT). The AAC are encoded mainly by the *aac(3)-II*, *aac(3)-IV*, and *aac(6')-Ib* genes, mostly located on IncF, IncI, and IncN plasmids, class 1 integrons, and transposons (Tn1331-like)¹⁰²⁻¹⁰⁷. These genes are globally disseminated among humans, animals, and environmental compartments^{102,105-108}.

The APH enzymes are encoded mostly by the *aph(6)-Id*, *aph(3'')-Ib* (alternatively named *strA* and *strB*), and *aph(3')-Ia* genes, the first two conferring streptomycin resistance, and the latter underling kanamycin resistance. The *strA* and *strB* genes are co-localized on diverse genetic elements (Tn3 and Tn5393) in Gram-negative species, similarly to the *aph(3')-Ia* gene associated with the insertion sequence IS26 on a transposon Tn5715^{109,110}.

The ANT enzymes are encoded by the *ant(2'')-Ia* and *ant(3')-Ia*, mainly. The *ant(2'')-Ia* gene confers resistance to gentamicin, tobramycin, and kanamycin and is integrated into class 1 integrons commonly reported from nosocomial, animal, and environmental isolates^{111,112}. The *ant(3'')-Ia* confers resistance to streptomycin in animal isolates and is located on Tn1331¹¹³⁻¹¹⁵.

More recently, the 16S rRNA methylation, impeding the interaction between aminoglycosides and the ribosome, has emerged. This mechanism is underlined by the *rmt*

family genes conferring pan-aminoglycosides resistance. The *rmtB* gene was reported for the first time in 2003 on a plasmid containing Tn3 right-end transposon in *Serratia marcescens*¹¹⁶. Several studies suggest that the dissemination of the *rmtB* gene is strongly linked to the dissemination of beta-lactam resistance genes in humans, in hospitals, and pets¹¹⁷⁻¹²¹.

Tetracyclines, similarly to aminoglycosides, target the 30S rRNA decreasing cell growth. Tetracycline resistance is conferred by the *tet* genes, which encode for efflux pumps, for instance, *tetA* and *tetB*, or ribosomal protection proteins, such as *tetM*. All of those genes are widely spread among Gram-negative bacteria and are found in all ecological niches¹²²⁻¹²⁴.

Phenicol consists of two antibiotics: chloramphenicol and its derivative florfenicol, which inhibit protein synthesis. Resistance mechanisms consist of chloramphenicol acetyltransferase encoded by the *catA* and *catB* genes, and efflux pumps encoded by the *floR*, *fexA*, and *cfr* genes. The *floR* gene is the most prevalent plasmid-located gene encoding resistance to both chloramphenicol and florfenicol in Enterobacterales¹²⁵.

Besides the mechanism of action and the related resistance, all sorts of antimicrobial therapy interact with the IM directly, after oral administration, or indirectly, after metabolization of the drug through biliary excretion^{126,127}. Effects of antibiotics on the bacterial composition of IM can be dramatic, with severe consequences on the host health. In the following chapter, the current knowledge on the interaction of antibiotics and IM will be reported, with a special focus on the bacterial composition and selection of AMR in calves, previous description of IM maturation and composition in humans and bovines.

2. The intestinal microbiota, a complex biome

2.1. History and technics of microbiota analysis

The human IM study started in the early 1670s with Antoine van Leewenhoek who described his own stool and saliva using the first microscope. He highlighted differences in microbes between these two samples and also between samples from healthy and diseased individuals¹²⁸.

A few centuries later, during the 1880s, Theodor Escherich discovered the first bacterium found in the intestines of diarrheic and healthy children that was named *Escherichia coli*¹²⁹. Since this first identification, numerous other bacteria were described at the end of the XIXth century, such as *Veillonella parvula* and bifidobacteria^{130,131}. In 1965, the

first microscopic image of bacteria in the mucosa in the gastrointestinal tract (GIT) in rodents was produced by Dubos *et al.*¹³². In the 1990s, Bocci demonstrated that the microflora residing in the gut had the same metabolic function as an organ, which plays a role in the health and disease of the host¹³³. In 2015 Marchesi and Ravel provided the most recent definition of the microbiome as an “entire habitat, including the microorganisms, their genomes, and the surrounding environmental conditions”¹³⁴.

Before the 1990s, microbiota analysis was limited to cultivated-based techniques. This approach allowed the identification of almost 20% of the global resident population contained in the gut¹³⁵. However, these techniques are considered time-consuming, labor-intensive and some bacterial species are not yet cultivable¹³⁶. Nowadays, after a period of decline, cultivation techniques are in constant evolution with new complex culture media which could potentially allow cultivation up to 30-60% of the GIT residents¹³⁷⁻¹³⁹. Most studies on the IM are focusing on the large intestine. Stools are considered representative of the large intestine and constitute non-invasive sampling^{140,141}. Each milliliter of the small intestine contains 10^8 microbial cells, raising in the large intestine to 10^{11} microbial cells. Overall the bacterial components of the GIT weights between one and two kilos¹⁴²⁻¹⁴⁴.

The development of the DNA sequencing platforms opened new possibilities to explore the IM and allowed a relative reduction in costs and laboratory procedures. This culture-independent method allowed deeper access to the global population of the gut, becoming a gold standard technique in microbiota research¹⁴⁵.

The first molecular approaches were based most commonly on the sequencing of the hypervariable region 4 of the 16S bacterial, ribosomal RNA gene^{146,147}. Bioinformatic analyses are conducted using tools organized in pipelines¹⁴⁸. The first shotgun metagenomic analysis was realized in 2006¹⁴⁹. The metagenomic approach requires more complex bioinformatics and computational efforts compared to the 16S rRNA sequencing. Nowadays, the number of pipelines dedicated to metagenomics is in constant evolution and is improving rapidly¹⁵⁰⁻¹⁵².

When comparing these two sequencing approaches, the 16S rDNA sequencing is known to be fast and cost-effective but this approach is limited on targeting the 16S rRNA gene, this amplification is biased notably by the number of copies of the gene, fluctuating among the bacterial species¹⁵³. Shotgun sequencing is more expensive and needs more effort and informatic skills for its analysis, but provides holistic knowledge on the IM composition including archaea, fungi, eukaryotes, and viruses, and also ARGs. This approach avoids amplification biases by using universal primers and enables *de novo* assembly of genomes by

assembly and binning of the generated reads¹⁵³. However, it gives information relative to the most represented genes due to its detection limit (10^8 reads per gene)¹⁵⁴. This limitation has been partially overcome by further bioinformatics analysis¹⁵⁵. Horizontally transferred and strain-specific genes may be essential within the GIT, in particular when they code for relevant functions such as antibiotic resistance or toxins^{156,157}. In this light, the prediction of potential functions or even metabolic features based on the genus-level taxonomic profiles, obtained for instance with the 16S gene sequencing, must be regarded as questionable^{158,159}.

For both approaches, important biases in the resulting bacterial community^{160,161} are introduced during sampling collection and conservation. The storage condition can induce DNA degradation compromising all the downstream analyses. Another important bias commonly recognized during sampling preparation is DNA extraction. For these reasons, the International Human Microbiome consortium has provided standards (IHMS) for samples collection, storage, and DNA extraction for microbiome sequencing and global comparison^{162,163}.

Overall, shotgun sequencing remains highly informative. The multiplication of studies on the human body sites allowed the creation of consortia such as the Human Microbiome Project and the Metagenomics of the Human Intestinal Tract (MetaHIT)^{164,165}. MetaHIT consists of a gene catalog containing 3.3 million unique genes from the human GIT microbiota. In 2014, an update of the catalog was performed via the addition of further biological samples creating a database from three-continent containing almost 10 million genes¹⁶⁶. Recently, other catalogs specific to mice¹⁶⁷, cattle^{168,169}, dogs¹⁷⁰, cats¹⁷¹ or pigs¹⁷² were developed. Nowadays, a combination of enhanced cultivation coupled with advanced sequencing approaches is allowing the characterization of organisms that are not cultivable at a traditional laboratory scale^{173,174} contributing to disclose the so-called 'dark matter' of microbiomes.

Another molecular technique typically used in the detection and quantification of specific genes is quantitative PCR (qPCR), which is very specific, sensitive, relatively inexpensive, and does not require sophisticated bioinformatic skills.

2.2. Composition of mammals' intestinal microbiota at early life

The mammal IM demonstrates conserved functions and composition along with the GIT. Furthermore, only 10-20% of the taxa constituting the IM differ among individuals and

are influenced by several factors including diet, geographic location, host genetics, birth delivery, age, that can play a positive and negative effect on the host health¹⁷⁵⁻¹⁷⁷. The core IM begins to be established after solid food introduction in the diet.

The GIT of mammal newborns provides an empty environment that is rapidly colonized by bacteria within the first day of life¹⁷⁸. This colonization is influenced by factors related to the host, such as luminal pH, food retention time, and immune defense mechanisms; by environmental factors, such as maternal microbiota, birth delivery, diet, housing, and antibiotic treatment during early life^{11,178-182}. Other factors are relative to bacteria abilities such as adhesion to epithelial cells, survival mechanisms under oxygen gradient, and mechanisms to obtain nutrients from the host.

Initial IM of full-term, human neonates born by vaginal delivery is dominated by Actinobacteria and by genera residents in the mother's vagina such as *Lactobacillus* and *Veillonella* of the Firmicutes phylum. Whereas in neonates born by cesarean section a lower species diversity and *Bifidobacterium* and *Bacteroides* richness are observed^{183,184}.

Similarly, calves IM at birth is characterized by low diversity and dominance by anaerobic facultative bacteria such as *Streptococcus*, *Escherichia* and *Enterococcus* genera to create enteric hypoxia to facilitate the colonization of anaerobic bacteria including Actinobacteria, Bacteroidetes, and Firmicutes phyla (majorly represented by Ruminococcaceae, *Prevotella*, Paraprevotellaceae, Lachnospiraceae, *Turicibacter*, *Succinivibrio*, Clostridiaceae, Bifidobacteriaceae and Bacteroidaceae families and genera)¹⁻³. The GIT of a pre-weaned calf is considered monogastric because the rumen is underdeveloped thus microbial colonization in the hindgut of calves is crucial. Here, plant fiber, oligosaccharides, and starch are fermented by the IM¹⁸⁸⁻¹⁹⁰.

In newborn mammals, IM evolves rapidly, with the bacterial abundance drastically increasing and diversifying¹⁹⁰⁻¹⁹⁵. At this moment nutrition is determinant in shaping IM¹⁹⁶. Human milk is composed of proteins, fats, and mainly galactooligosaccharide, together with almost 200 bacterial species belonging to the genera *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Weissella*, and *Bifidobacterium*^{183,197-199}. Bifidobacteria, and few *Bacteroides* species, including *B. thetaiotaomicron* and *B. fragilis*, can degrade the galactooligosaccharide directly by fermentation in short-chain fatty acids^{183,200,201}. In particular, the Human Milk Oligosaccharides (HMOs) promote the colonization by *Bifidobacterium infantis*, resulting in anti-inflammatory effects and diarrhea prevention^{202,203}.

In pre-weaned calves feeding methods are numerous. A common practice is the consumption of colostrum, which is dominated by *Lactococcus*, *Staphylococcus*, *Pseudomonas*, *Streptococcus*, and *Escherichia* genera and is presenting a beneficial effect on the GIT calf later in life²⁰⁴. Colostrum feeding promotes colonization of beneficial bacteria in the small intestine, weight gain and intestinal development (e.g. villus circumference, height and epithelial cell proliferation) of neonatal calves^{182,205,206}. In the neonatal calf intestine also anaerobic fibrolytic fungi are reported including *Caecomyces*, *Orpinomyces*, and also phages with the *Microviridae* family as the most prevalent¹⁹⁵. Later, milk feeding provokes a microbial succession to lactose-degrading species such as the archaeal genus *Methanobrevibacter*^{195,207}. The lactose fermentation will release hydrogen and carbon dioxide required for methanogenesis²⁰⁸.

Besides nutrition, the interaction between human newborns and their relatives may influence the primordial microbial colonization^{179,188}. In calves, IM is more similar to environmental microbiota than to IM of their mothers²⁰⁹, probably because the newborn calf is exposed to the soil, bedding material, and other farms' components immediately after birth. On the other way, protozoa colonization of the intestine is based on indirect contact with the saliva of adult animals. Indeed, when calves are separated from their dams right after birth and kept isolated until weaning, these dairy calves remain naturally protozoa-free²¹⁰.

2.3. Intestinal microbiota at the adult age in humans

The introduction of solid food in the diet results in the complexification of metabolites found in the gut, which increases the diversity of bacterial species towards an adult-like, complex IM. The composition of the adult IM, in humans, varies among geographical location, cultures, and thus diet style²¹¹. Non-Western diets promote bacterial species richness in the gut²¹¹, because they contain more fibers and less meat, sugar, and fat. Additionally, hygiene, exposure to animals and environments, or enteric parasites contribute to the differences observed in the IM between inhabitants of low/medium-income countries and high-income countries²¹².

In the core of an adult IM up to 1000 distinct species have been reported, belonging to six phyla notably dominated by the anaerobic Bacteroidetes and Firmicutes, followed by Proteobacteria, Actinobacteria, Verrucomicrobia, and Fusobacteria¹⁷⁶.

Within the phylum, Bacteroidetes, *Bacteroides* and *Prevotella* genera are the most predominant organisms^{213,214}. These genera are able to digest complex polysaccharides that

cannot be digested by the hosts. Furthermore, species of the *Prevotella* genus are major producers of propionate, which has an anti-inflammatory role and is involved in energy homeostasis by increasing sensitivity to insulin^{194,215}. In the *Bacteroides* genus, some species are considered also major butyrate producers. Butyrate has a non-negligible role in the gut physiology, immunity and inflammatory response by enhancing the gut barrier via upregulation of genes encoding tight junctions (e.g., claudion-1, Zonula, Occludens-1), regulation of T-cells, Toll-like receptors (TLR) activation, and inhibition of cytokines. All these functions have been observed in humans and calves, as well^{175,216–219}.

The phylum Firmicutes contains both obligate and facultative anaerobic bacteria. Most members are Gram-positive and can form endospores, favoring survival under stressful environmental conditions. The Clostridia class is the largest group belonging to the phylum, containing numerous bacterial species that preferentially colonize the mucosal folds, establishing a close relationship with the intestinal epithelial cells contributing to their homeostasis²²⁰. Among Clostridia, *Faecalibacterium prausnitzii* is a relevant butyrate producer²²¹. Species of the genus *Veillonella* include organisms able to produce propionate, whereas lactobacilli preferentially ferment sugars to lactate and acetate^{194,222}.

The phylum Actinobacteria is composed of Gram-positive, both aerobic and anaerobic, bacteria. The most dominant, genus found in the GIT consists of *Bifidobacteria*^{213,223}, which are major producers of acetate^{203,224}.

Proteobacteria phylum includes facultative and obligate anaerobes, Gram-negative bacteria, commonly found along with the GIT²²⁵. These bacteria are in low abundance in a “healthy” IM, except in infants fed principally by milk. Several studies suggest that increased levels of Proteobacteria are considered as a signature of dysbiosis and risk of disease²²⁶. Within the phylum Proteobacteria, the most common enteric pathogens belong to Enterobacterales. When the IM is insulted, the Enterobacterales abundance blooms and can cause infections and inflammatory response²²⁷.

The phylum Verrucomicrobia is largely represented by *Akkermansia. muciniphila* within the human and animal IM. *A. muciniphila* is a Gram-negative, non-mobile, and anaerobic bacterium able to produce propionate via mucin degradation²²⁸.

The phylum Fusobacterium includes anaerobic, elongated, Gram-negative filamentous bacteria, whose most species are typically associated with human and animal disease. For instance, *F. necrophorum* is an opportunistic pathogen that causes numerous necrotic conditions (necrobacillosis) and both specific and non-specific infections in a variety of

animals. Several clinical studies on human inflammatory bowel disease (IBD) suggest a link of the disease with *F. varium*²²⁹.

When the host-microbiota reaches its stable community, no dramatic changes will occur spontaneously. In the study conducted by Faith *et al.*, analyzing the IM of thirty-seven healthy adults during five years, half of the bacterial strains detected at the beginning of the study persisted throughout the entire study period²³⁰.

2.4. Intestinal microbiota at the adult age in bovines

During weaning, differences between humans and bovines are more evident. In this phase, solid intake in calves increases the fibrolytic and methanogenic microorganisms' abundance in the IM, but also SCFAs (Short-Chain Fatty Acid) level within the rumen, along with a decrease of ruminal pH^{195,231,232}. This passage promotes a cascade of changes in the GIT resulting in the development of the rumen papillary size, of the intestinal epithelium, which provides the niche for colonization to further microbial species. Certain farm management is forcing the rumen maturation using specific nutrition for weaning acceleration and weight gain^{233,234}. A consensus is to encourage the use of digestible starters to promote rumination²³⁵. The IM of calves raised with starters and mixed diets diversify with age²³⁶ and demonstrates a dominance of *Prevotella* and *Bacteroides* among bacteria, and of *Megasphaera* among archaea^{195,237}.

Weaned calves and adult bovines have an IM mostly composed of Firmicutes, Bacteroidetes (around 90% of the IM), Spirochaetes, and Fibrobacteres, which are rich in microorganisms possessing fiber-digesting enzymes^{238,239}. Some bacterial genera are capable of degrading complex fiber²⁴⁰, for instance, *Prevotella*, *Paraprevotella*, *Butyrivibrio*, *Treponema*, *Acetivibrio*, *Sporobacter*, *Coprococcus* and *Fibrobacter* as well as *Oscillibacter*, are markers of an adult-like IM.

Fungi also play a role in fibrous plant digestion and facilitate the fiber particle release within the rumen. They also produce a wide range of enzymes to ferment complex carbohydrates, releasing great amounts of hydrogen gas that favor the archaeal community^{241,242}. Therefore, anaerobic fungal activity may shape the structure of the bacterial and archaeal communities in the mature rumen, and consequently, influence fiber utilization efficiency and methanogenesis²⁴³.

Methanogenic Archaeae have also a role in the removal of hydrogen gas, which may constrain microbial growth and carbohydrate degradation at high levels along with the GIT²⁴⁴.

Ciliate protozoa have also an impact on nutrient digestibility, fermentation and methanogenesis²⁴⁵.

In the beef and dairy industries, intervention on the microbiota has been linked to changes in milk production, weight gain, and methane emissions^{246,247}.

3. Effect of antibiotic therapies on the mammals' intestinal microbiota

The IM is challenged by multiple environmental stresses including diet, toxins, drugs, and pathogens, which can provoke an imbalance in the microbial populations termed “dysbiosis”^{226,248}. This state is characterized by a profound decrease of bacterial diversity and, in particular, an increase in Bacteroidetes and pro-inflammatory pathogens, and a decrease of Firmicutes^{226,249}.

Besides causing dysbiosis, selective pressure exerted by antibiotics kills susceptible isolates giving an advantage to the antibiotic-resistant isolates driving the spread of ARGs in the IM¹. ARG, especially those found on mobile genetic elements (MGE) can persist in the IM for years after antibiotic therapies^{201,250,251}.

3.1. Effect of antibiotic therapies on the human intestinal microbiota

Investigations on the effect of antibiotics on the human IM have principally focused on beta-lactams and quinolones that are commonly used in human medicine for treating infections caused by Gram-negative bacteria. Studies have been conducted in adults with a mature IM that was not affected by an enteric disease or disorder (Table 1).

Effects of oral or intramuscular injection of penicillins were investigated in four studies^{252–255}, by the cultivation of bacteria on selective media. No variation in the relative abundance of species was observed before and after treatment, and no rise of ARGs related to penicillins resistance was reported^{252–255}. However, bacterial cultivation highlighted that ticarcillin and piperacillin within the Firmicutes phylum caused the replacement of *Clostridioites* and *Lactobacillus* genera by *Enterococcus* which include potentially pathogen isolates²⁵⁵.

Oral administration of amoxicillin^{256–259} and ampicillin²⁶⁰ revealed a deeper impact on IM composition. Bacterial abundance variation was observed by cultivation, resulting in a decrease in the abundance of Enterobacterales and some families of the Firmicutes phylum, which were replaced by Non-*E. coli* Enterobacterales, *Bifidobacterium*, *Bacteroides*, and *Eubacterium*^{256,257}. Quantification of the beta-lactamase activity was evaluated, as well, highlighting at the end of the antibiotic treatment an increase of this enzymatic activity^{256,257}.

Effect of amoxicillin associated with clavulanate acid^{261–265} administered intravenously²⁶¹ or orally^{262–265} was analyzed by cultivation^{261,264} or by 16S rRNA gene sequencing^{262,263,265} and demonstrated, for both administrations route, a global decrease of *Bifidobacterium* and *Clostridioites* genera were followed by an increase of Enterobacterales. Moreover, amoxicillin-clavulanic acid is able to persist for a long time within the gut continuing selective pressure on the IM even after therapy withdrawal²⁶⁴.

The effect related to cephalosporins therapy has been the most extensively investigated, so far^{266–271}. All these studies were based on bacterial cultivation except one study on cefotaxime in which 16S rDNA sequencing²⁷² was implemented. Besides, in this study, AMR was not analyzed. Third and fourth-generation cephalosporins caused an increase of Enterococcaceae, Non-*E. coli* Enterobacterales, especially *Klebsiella* spp., and a decrease in *Bacteroides*, *Clostridioites*, *Eubacterium*, *Fusobacterium*, *Lactobacillus*, and *Escherichia* genera. Similar effects were observed with first and second-generation cephalosporins, with an increase of *Bacteroides*, and a decrease in other genera such as *Bifidobacterium*, *Clostridioites*, *Eubacterium*, *Lactobacillus*, and *Escherichia*.

Studies investigating carbapenems' effects on the IM were based on bacterial cultivation^{273–276}. Multiples antibiotics and posology were tested (intravenous injection of 0,5g of meropenem for one week²⁷⁵; intravenous injection of 0,125g of lenapenem²⁷⁴; 3g daily of ritipenem axetil for 10 days²⁷⁶ and intravenous injection of 1g of ertapenem for one week²⁷⁶). Their influences on the IM composition resulted in an increase of *Enterococcus* genus and a decrease of Bacteroidetes and Firmicutes phyla, represented by *Bacteroides*, *Clostridioites*, *Streptococcus*, and *Veillonella* genera. No impact was highlighted on the ARGs related to carbapenems.

On the contrary, tetracyclines (doxycycline^{277,278} and minocycline²⁷⁹) influenced the amount of tetracyclines resistance genes and a general decrease of the bacteria amount, especially concerning the *Enterococcus* genus^{277–279}. Doxycycline was administered orally

^{277,278} and was evaluated by cultivation approach whereas oral administered minocycline impact was investigated by metagenomic²⁷⁹.

Similarly, fluoroquinolones (ciprofloxacin, norfloxacin, gemifloxacin, trovafloxacin, levofloxacin, and moxifloxacin) cause a global decreased in bacterial abundance belonging to the four major phyla, notably Firmicutes, which was deeply affected, Actinobacteria (*Bifidobacterium* and *Corynebacterium*), Bacteroidetes (*Bacteroides*), and Proteobacteria (*Escherichia*)^{279–291}. However, an increase of Enterobacterales, notably for *Citrobacter*, *Klebsiella*, and *Enterobacter* genera were highlighted. The quinolones therapy was also associated with an increased amount of genes conferring resistance to quinolones. These observations derive from studies majorly based on bacterial cultivation and with some exception on next-generation sequencing^{279,281,284,286}.

Sulfonamides and trimethoprim effects were investigated in three studies via bacterial cultivation approach^{292–294}. The drugs were oral-administered with different posology (trimethoprim 0,4g per day for four weeks²⁹²; trimethoprim-sulfamethoxazole 40mg + 200mg daily for a month²⁹⁴ and sulphasomidine 4g per day or sulphalene 0,1g per day during ten days²⁹³). The effects on the IM seemed limited to *E. coli* abundance, which decreased in favor of resistant isolates that could proliferate^{292–294}.

Globally, in adults, the IM has been reported to recover, in terms of restoring to the pre-therapy composition, from a single antibiotic exposure within approximately two weeks, but multiple, or long-lasting treatments can cause this time frame to expand substantially^{250,295–298}. However, even short-term therapies can perturb the IM composition for years in humans and also in other animals^{212,250,298}. This perturbation has a significant effect on microbial metabolic functions. Resilience can be reached by functional redundancy of bacterial species, which restores both stability and health of the IM^{144,174}.

Table 1. Summary of the studies analyzing the effect of antibiotics on the human intestinal microbiota.

Antibiotic therapy	Resistance selection	Effect on bacterial abundance	References
Penicillin	Not	None	252–255
Piperacillin and ticarcillin	Not	Firmicutes (Enterococcaceae↑, Clostridiaceae↓, Lactobacillaceae↓)	255
Amoxicillin and ampicillin	Yes	Actinobacteria (Bifidobacteriaceae↑), Bacteroidetes (Bacteroidaceae↑), Firmicutes (Lachnospiraceae↓, Veillonellaceae↓, Eubacteriaceae↑), Proteobacteria (Enterobacteriaceae↑, <i>Escherichia</i> ↓)	252,255–259,279,299–303
Amoxicillin/clavulanate	Yes	Actinobacteria (Bifidobacteriaceae↓), Firmicutes (Clostridiaceae↓), Proteobacteria (Enterobacteriaceae↑)	261–265
1 st and 2 nd generation cephalosporins	Yes	Actinobacteria (Bifidobacteriaceae↓), Bacteroidetes (Bacteroidaceae↑), Firmicutes (Enterococcaceae↑, Clostridiaceae↓, Eubacteriaceae↓, Lactobacillaceae↓), Proteobacteria (Enterobacteriaceae↑, <i>Escherichia</i> ↓)	254,255,258,268,303,304
3 rd , 4 th , 5 th generation cephalosporins	Yes	Bacteroidetes (Bacteroidaceae↓), Firmicutes (Enterococcaceae↑, Clostridiaceae↓, Eubacteriaceae↓, Lactobacillaceae↓), Fusobacteria↓, Proteobacteria (<i>Klebsiella</i> ↑, <i>Escherichia</i> ↓)	255,259,266,267,269,271,272,276,303,305–314
Carbapenems	Not	Bacteroidetes (Bacteroidaceae↓), Firmicutes (Enterococcaceae↑, Clostridiaceae↓, Streptococcaceae↓, Veillonellaceae↓)	273–276
Tetracyclines	Yes	Firmicutes (Enterococcaceae↓)	277–279
Fluoroquinolones	Yes	Actinobacteria (Bifidobacteriaceae↓, Corynebacteriaceae↓), Bacteroidetes (Bacteroidaceae↓), Firmicutes↓, Proteobacteria (Enterobacteriales↑, <i>Escherichia</i> ↓)	279–291,295
Sulfonamides	Yes	Proteobacteria (<i>Escherichia</i> ↓)	292–294

Note: ↑ increase; ↓ decrease

3.2. Effect of antibiotic therapies on the bovine intestinal microbiota

The most common reasons for administering antibiotics to adult bovines are bacterial metritis or mastitis, and for preventing other infections^{315–317}. The effect of antibiotic therapies on bovine IM has been less extensively investigated compared to humans. Current reports on this issue also include the effect of antibiotic residues contained in the waste milk used as food for calves.

Penicillins are the most used antibiotics for treating bovines in Northern Europe. This antibiotic is able to increase phenotypic resistance to multiple unrelated antimicrobial agents among fecal *E. coli* and also induce changes in the IM in calves³¹⁸. The association of penicillin and streptomycin was analyzed by 16S rDNA sequencing of the IM from the foregut and hindgut of treated calves³¹⁹. A notable decrease of the Firmicutes phylum was observed in both compartments for both antibiotics compared to the initial condition. No analysis was performed on the development of AMR.


Similarly, oxytetracycline, which is commonly used for treating respiratory diseases in cattle in the US, plays a role in the nasopharyngeal and IM community with a decrease in the abundance of Prevotellaceae, Bacteroidaceae, Rikenellaceae, and Sutterellaceae, whereas an increase of *tetM* and *tetW* genes was observed by qPCR³²⁰. In another geographical context, oxytetracycline did not demonstrate a significant effect on the IM composition of calves although the antibiotic was administered for a longer duration and orally. However, an increase of the *tetM* and *floR* genes was observed³²¹.

Recently, Dobrzanska *et al.* studied the effect of florfenicol to assess the effects on the resistome and the IM composition in calves, in the UK. Fecal samples were analyzed by shotgun sequencing and revealed that the antibiotic increased the proportion of Proteobacteria, principally *E. coli*, and caused a decrease of Prevotellaceae. Analysis on ARGs highlighted the increase of the *mcr-2* amount, linked to the increase of *E. coli*³²².

Fluoroquinolones, including enrofloxacin, are considered critical antibiotics for human medicine to be avoided in animals when alternatives are available. However, they are used to treat bovine respiratory disease in the US. It appears, according to shotgun sequencing analysis, that no changes were induced by enrofloxacin in the IM of treated calves³²³.

Besides the therapeutic context, antibiotics even in low concentration can reach the animals' intestine by food. An example is provided by non-saleable milk from antibiotic-treated lactating cows. Often, residues of antibiotics occur as a cocktail. In one case, ceftiofur, penicillin G, and oxytetracycline were found contemporaneously and caused a decrease in

Veillonellaceae, Streptococcaceae, and Clostridiaceae families abundance according to 16S rDNA sequencing³²⁴. A similar antibiotic cocktail than the aforementioned, with the addition of streptomycin, caused an increase of species belonging to the *Prevotella* genus³²⁵. Both investigations on antibiotic residues in milk for calves were based on the 16S rDNA sequencing and no information on the resistome could be gained.



II. Aim and objectives of the study

Amoxicillin is one of the most used antibiotics worldwide in humans for treating non-complicated infections. In veterinary medicine, amoxicillin is considered as an antibiotic to be used with prudence and when possible, as first-line treatment. Preserving its activity is thus of global health importance. The effect of oral-administered amoxicillin on IM has been investigated in humans, highlighting a change in the Enterobacterales composition with an increase of non-*E. coli* species and decrease of certain genera of the Firmicutes phylum (*Lachnospiraceae*, *Lactobacillaceae*...). Amoxicillin treatment increased beta-lactamases activity in the IM of treated patients, suggesting selection of beta-lactamases encoding genes.

Despite the clinical importance and the large usage of amoxicillin in veterinary medicine, longitudinal studies analyzing the effect of amoxicillin treatment on the IM of calves are lacking. To fill in this gap of knowledge, the effect of amoxicillin treatment was evaluated in a group of calves hosted in commercial farms and suffering from omphalitis. This condition is known for not having consequences on the IM composition, but it requires antibiotic treatment.

Stools were prospectively collected from calves' (n = 12) rectum before amoxicillin treatment (T0), at amoxicillin therapy withdrawal (T1), and one week after amoxicillin withdrawal (T2). Amoxicillin was administered to calves by intra-muscular injections. At the same pace of treated calves' sampling, stools were collected from a control group of calves (n = 5) not receiving amoxicillin therapy. Calves of the control group lived on the same farms of the treated ones (Table 2). All farms were located in the Rhône-Alpes region. Stools were then analyzed according to three methodological axes:


- 1) quantification by qPCR in DNA extracted from stools of most common ARGs encountered in Enterobacterales and comparison of their amount at the three samplings and between treated and untreated calves. This methodology will permit to analyzed occurrence and amount of ARGs with high specificity.
- 2) cultivation of viable Gram-negative bacteria, in aerobic conditions, for comparing the amount of amoxicillin resistant viable bacteria at the three samplings, between treated and untreated calves; characterization of the isolates by whole-genome sequencing to analyze the presence of ARGs, supporting genetic elements, clonality and genetic relatedness and virulence factors. This axe will permit to elucidate eventual persistence, circulation of clones and, possibly, ARGs shuffling.

3) shotgun sequencing of the DNA extracted from stools to analyze the effect of amoxicillin treatment on the overall IM composition, resistome and relative genetic elements, together with potential bacterial functions in the community.

Methodologies proper to the development of the tree axes are detailed in the papers that have been published, submitted or in preparation reported in the following section:

Table 2. Features of calves enrolled in the study and details of the amoxicillin treatment.

Calf information					Amoxicillin treatment				
ID	Farm	Breed	Sex	Age (days)	Start (T0)	End (T1)	7 days post T1 (T2)	Number of injections	Volume of antibiotic (15 mg/animal Kg)
2	A	Charolais/ Montbeliard	F	8 d	07/10/18	14/10/18	21/10/18	4	6 mL
3	B	Charolais/ Montbeliard	M	8 d	08/10/18	29/10/18	05/11/18	8	6 mL
4	B	Montbeliard	M	6 d	12/10/18	29/10/18	05/11/18	8	6 mL
5	B	Montbeliard	F	5 d	12/10/18	29/10/18	06/11/18	8	6mL
7	C	Charolais/ Montbeliard	M	18 d	13/06/19	18/06/19	25/06/19	3	5 mL
9	C	Prim' Holstein	M	13 d	13/06/19	18/06/19	25/06/19	3	5 mL
10	C	Charolais/ Prim' Holstein	F	7 d	13/06/19	18/06/19	25/06/19	3	5 mL
11	D	Limousin/ Montbeliard	M	7 d	30/07/19	08/08/19	15/08/19	4	4 mL
12	D	Charolais/ Montbeliard	M	6 d	30/07/19	08/08/19	15/08/19	4	4 mL
13	D	Charolais/ Montbeliard	F	5 d	30/07/19	08/08/19	15/08/19	4	4 mL
14	E	Prim' Holstein	F	10 d	30/07/19	02/08/19	09/08/19	2	5 mL
15	F	Prim' Holstein	F	26 d	05/08/19	12/08/19	19/08/19	4	5 mL
16	C	Montbeliard	F	8 d	13/06/19	18/06/19	25/06/19	-	-
17	E	Prim' Holstein	M	5 d	30/07/19	02/08/19	09/08/19	-	-
18	G	Montbeliard	M	7 d	06/03/20	13/03/20	20/03/20	-	-
19	A	Montbeliard	F	8 d	06/03/20	13/03/20	20/03/20	-	-
20	A	Charolais/ Montbeliard	M	7 d	06/03/20	13/03/20	20/03/20	-	-



III. Results and publications

1. Effect of amoxicillin treatment on the antibiotic resistance genes abundance in calves' intestinal microbiota.

For the evaluation of ARGs abundance in stools of calves, qPCR assays targeting the major ARGs found in Enterobacterales in animals were developed. The assays included ten genes conferring resistance to beta-lactams, twelve to aminoglycosides, three to fluoroquinolones, three to tetracyclines, two to colistin, and six to sulfonamide-trimethoprim. The assays included also genes evocative of integrons' presence, *intI1/2/3*, as index of multidrug resistance development. The panel comprised also an assay for the quantification of a conserved part of the 16S rDNA. Specificity, efficiency, limit of quantification, and limit of detection were evaluated.

These assays may be useful for tracking and quantifying AMR in various contexts and Gram-negative bacteria. Implementation of further targets in the panel is simple and requires limited optimization processes.

The methodology was part of a publication in the journal "Diagnostic Microbiology and Infectious Disease" (<https://doi.org/10.1016/j.diagmicrobio.2021.115328>).



Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Original Article

An inventory of 44 qPCR assays using hydrolysis probes operating with a unique amplification condition for the detection and quantification of antibiotic resistance genes

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ARTICLE INFO

Article history:

Received 3 September 2020

Revised in revised form 22 January 2021

Accepted 24 January 2021

Available online 30 January 2021

Keywords:

TaqMan
Genotyping
*bla*_{CTX-M}
*bla*_{OXA}
mcr1-3
16S rDNA

ABSTRACT

Early antibiotic resistance determinants (ARDs) detection in humans or animals is crucial to counteract their propagation. The ARDs quantification is fundamental to understand the perturbation caused by disruptors, such as antibiotics, during therapies. Forty-three qPCRs on the most diffused ARDs and integrons among human and animal *Enterobacterales*, and one on the 16S rDNA for bacteria quantification, were developed. The qPCRs, using hydrolysis probes, operated with a unique amplification condition and were tested analytically and diagnostically performing 435 reactions on five positive and negative controls for each qPCR. Diagnostic sensitivity and specificity were confirmed by PCR and genome sequencing of control isolates, demonstrating 100% performance for all qPCRs. An easy and rapid discrimination method for the epidemiologically relevant *bla*_{CTX-Ms} is provided. This large, noncommercial qPCRs inventory could serve for precise quantification of ARDs, but also as a rapid screening tool for surveillance purposes, providing the basis for further high-throughput developments.

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1. Introduction

Antimicrobial resistance (AMR) is a threat to global health for both humans and animals (Appelbaum, 2012; O' Neill, 2016). Antibiotics are able to select and amplify antibiotic resistance determinants (ARDs) in the host, favoring their dissemination widely (Casals-Pascual et al., 2018; Sullivan et al., 2001). Antibiotics have different selective effects (Ianiro et al., 2016), thus quantifying ARDs before and after antibiotic therapy usefully reveals which molecules may have limited selective impacts on the resistome. Furthermore, early detection of ARDs in hosts or the environment is crucial to temporally develop hygienic measures avoiding further dissemination, to this end rapid molecular approaches are required.

Polymerase chain reactions (PCR) remain the most propagated methodologies in clinical and research laboratories, especially in low or medium income countries. In particular, qPCR assays, based on hydrolysis probes, commercially known as TaqMan, are rapid closed-tube tools, diagnostically specific, that also allow accurate quantification of the targets of interest. Hydrolysis probes hybridize on the host

complementary DNA sequence and emit fluorescence during the amplification process when the Taq polymerase removes, by hydrolysis, the fluorophore at 3'-end, which will emit a fluorescence signal once far from the quencher at the 5'-end of the probe (Lupo et al., 2013). Quenchers with minor groove binding (MGB) motifs enhance the probe melting temperature, allowing to design short length probes while enhancing specificity. Commercially available qPCRs on ARDs exist, but they are based exclusively on ARDs of human clinical concern and, overall, few developments have included other antibiotic classes than beta-lactams.

In this study, qPCRs using hydrolysis probes for *int1/int2* integron genes together with 42 of the most diffused ARDs in *Enterobacterales* according to the current AMR epidemiology were designed (Poirel et al., 2018). Integrons have been recurrently proposed as relevant indicators of multidrug resistance (Amos et al., 2015; Leverstein-van Hall et al., 2003) whereas the 42 ARDs confer resistance to beta-lactams, aminoglycosides, fluoroquinolones, colistin, folic acid pathway inhibitors, tetracyclines, and phenolics, common both to human and veterinary medicine. One assay was also designed to possibly quantify ARDs relatively to the bacterial abundance estimated by the amplification of a conserved region of the 16S rDNA.

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2. Materials and methods

2.1. Primers and probes design

Primers (Sigma Aldrich, France) and probes (TaqMan, Applied Biosystem, France) were designed using the Primer Express v.3.0.1 software (Applied Biosystem, California) in order to: (1) avoid primers dimers and with a melting temperature (Tm) 10°C lower than the probe (intended at 70°C); (2) obtain the same *in silico* amplification conditions for all qPCRs; (3) allow the possibility of multiplexing; (4) discriminate the variants of family genes (e.g., *bla*_{CTX-M} groups and variants) with genotyping assays. For the SNPs visualization, a ClustalW alignment was performed on the nucleotide sequences using BioEdit v. 7.2.5 software. The nucleotide sequences and accession number for each gene were retrieved from the Genbank database (Tables 1–3).

Probes were labeled with a fluorophore (JUN, or VIC, or ABY, or FAM) at the 3'-end and a quencher (MGB or QSY) at the 5'-end.

2.2. Reaction conditions of qPCRs

The qPCR reactions were performed in 384-wells plates in 5 μ L final volume containing 2.5 μ L of TaqPath ProAmp Multiplex Master Mix (Applied Biosystem, Lithuania), 0.5 μ L of forward and reverse primer (1 μ M), respectively, 0.25 μ L of TaqMan probe (50 nM), 1.25 μ L of sterile H₂O and 1 μ L of DNA. For the genotyping assays, primers

were used at 200 μ M final concentration, whereas probes (only with MGB quencher) were used at 100 μ M (Malkki et al., 2012).

For both qPCRs and genotyping assays, amplification was performed in fast mode using QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific) (Table S1). Data were collected at the annealing-amplification step and analyzed using the QuantStudio Design & Analysis Software v1.4.3. (ThermoFisher Scientific).

2.3. Efficiency and limit of quantification

The efficiency of qPCRs was estimated performing a calibration curve. For each gene, a PCR product was obtained using the same primers of the qPCR assays. PCR products were purified (NucleoSpin Gel and PCR Clean-up, Machery-Nagel, Germany) and quantified by spectrophotometer absorbance and fluorometry (NanoDrop One, Ozyme, France, and Qubit 4 Fluorometer, Thermo Scientific, France) then freshly diluted (1:10 dilutions, $n = 12$, from 10 ng/ μ L) and used in technical replicates. For each gene assay, three nontemplate controls were generated, whereas three replicates contained 10 ng of DNA from ARD-free *E. coli* CIP 76.24. The mean Cq values were plotted against the respective gene copies number (Lee et al., 2006), generating a regression curve. The efficiency (E) was calculated using the slope from the regression curve ($E = 10^{(-1/\text{slope})} - 1$) and values between 90% and 110% were defined as acceptable (Bustin et al., 2019). The limit of quantification (LoQ) was determined according to CLSI recommendations as the Cq obtained using the highest dilution

Table 1

Primes and probes (TaqMan, Applied Biosystem) sequences of 17 qPCRs on beta-lactams resistance genes and features of positive ($n = 63$) and negative control isolates ($n = 40$). Genetic content of the isolates was determined by PCR or genome sequencing (underlined isolates) and ResFinder 3.2 analysis.

Gene, Accession number	Primer F, R; probe sequence (5'→3')	Amplicon size (bp)	Isolate containing the target (variant) and other genes relative to beta-lactams resistance	Isolates target-free and other genes relative to beta-lactams resistance ^a
<i>bla</i> _{TEM-1} Y13612.1	F: TTGCACACATGGGGGATC R: AGCTAGAGTAAGTAGTTCGCCAG-TTAATAGTT QSY-AAACCGGAGCTGAATGAAGCCA-TACCAA-ABY	153	<u>Ec_58</u> (<i>bla</i> _{TEM-190}), <i>bla</i> _{CTX-M-1} 20071 (Madec JY et al., 2012) (<i>bla</i> _{TEM-1C}), <i>bla</i> _{CTX-M-15} , <i>bla</i> _{OXA-1} <u>Ec_50</u> (<i>bla</i> _{TEM-18}), <i>bla</i> _{CTX-M-14} , <i>bla</i> _{OXA-1} <u>Ec_41</u> (<i>bla</i> _{TEM-1A}), <i>bla</i> _{CTX-M-14} <u>Ec_60</u> (<i>bla</i> _{TEM-52})	<u>Ab_96</u> : <i>bla</i> _{SHV-182} , <i>bla</i> _{OXA-1} , <i>bla</i> _{OXA-1} <u>KE_105</u> : <i>bla</i> _{SHV-1307} , <i>bla</i> _{OXA-1} , <i>bla</i> _{OXA-1} <u>Ab_90</u> : <i>bla</i> _{OXA-23} <u>Ec_43</u> : <i>bla</i> _{CTX-M-55}
<i>bla</i> _{SHV} AJ920369.1	F: CGATAACAGCGCCGCC R: TTCCCAGCGGTAAGGC QSY-TGACTGCTTTTGGCCAGATCG-JUN	109	<u>Ec_27</u> (<i>bla</i> _{SHV-5}) <u>Kp_98</u> (<i>bla</i> _{SHV-76}), <i>bla</i> _{TEM-18} , <i>bla</i> _{CTX-M-15} <u>36250</u> (Diab M et al., 2017) (<i>bla</i> _{SHV-12}) <u>Kp_99</u> (<i>bla</i> _{SHV-182}), <i>bla</i> _{OXA-1} , <i>bla</i> _{OXA-1} <u>Ec_104</u> (<i>bla</i> _{SHV-12}), <i>bla</i> _{OXA-1} , <i>bla</i> _{OXA-1}	<u>Ec_39</u> : <i>bla</i> _{CTX-M-15} , <i>bla</i> _{TEM-18} <u>Ec_50</u> : <i>bla</i> _{CTX-M-14} , <i>bla</i> _{TEM-18} , <i>bla</i> _{OXA-1} <u>Ec_110</u> : <i>bla</i> _{CTX-M-1} , <i>bla</i> _{TEM-1A} <u>Ec_04</u> : <i>bla</i> _{CMY-2} , <i>bla</i> _{TEM-18}
<i>bla</i> _{CTX-M-1-ep} X92506.1	F-CTGGGTGTGGCATTGATTAACA ^d R-CTGCCTGATTTAAGACGATTCGGTT ^d QSY-TGATGAGCGCTTGGCATGTGCA-ABY	151	<u>Ec_58</u> (<i>bla</i> _{CTX-M-1}), <i>bla</i> _{TEM-190} <u>Ec_39</u> (<i>bla</i> _{CTX-M-15}), <i>bla</i> _{TEM-18} <u>Ec_17</u> (<i>bla</i> _{CTX-M-32}) <u>27841</u> (Haenni M et al., 2018) (<i>bla</i> _{CTX-M-55}), <i>bla</i> _{TEM-1} <u>Ec_43</u> (<i>bla</i> _{CTX-M-55}) <u>Ec_61</u> (<i>bla</i> _{CTX-M-1})	<u>Ec_04</u> : <i>bla</i> _{CMY-2} , <i>bla</i> _{TEM-18} <u>Ec_82</u> : <i>bla</i> _{CTX-M-2} <u>Kp_97</u> : <i>bla</i> _{SHV-182} , <i>bla</i> _{OXA-1} , <i>bla</i> _{OXA-1} <u>Ec_14</u> : <i>bla</i> _{CTX-M-8}
<i>bla</i> _{CTX-M-1ep-2397} ^d X92506.1	MGB-ATGCCCGTGGCCG-FAM	151	<u>Ec_61</u> (<i>bla</i> _{CTX-M-1}) <u>27259</u> (Lupo A et al., 2018a) (<i>bla</i> _{CTX-M-1}) <u>27732</u> (Lupo A et al., 2018b) (<i>bla</i> _{CTX-M-55}) <u>32137</u> (Lupo A et al., 2018a) (<i>bla</i> _{CTX-M-1}) <u>Ec_31</u> (<i>bla</i> _{CTX-M-55})	<u>Ec_71</u> : <i>bla</i> _{CTX-M-15} <u>Ec_16</u> : <i>bla</i> _{CTX-M-15} <u>Ec_20</u> : <i>bla</i> _{CTX-M-15} <u>24424</u> (Dahmen S et al., 2013): <i>bla</i> _{CTX-M-3} , <i>bla</i> _{TEM-18}
<i>bla</i> _{CTX-M-1ep-239C} ^d Y10278.1	MGB-CCAGTAAAGTGATGCCCGC-VIC	151	<u>Ec_71</u> (<i>bla</i> _{CTX-M-15}) 20071 (Madec JY et al., 2012) (<i>bla</i> _{CTX-M-15}), <i>bla</i> _{TEM-1C} , <i>bla</i> _{OXA-1} <u>Ec_16</u> <u>24424</u> (Dahmen S et al., 2013) (<i>bla</i> _{CTX-M-3}), <i>bla</i> _{TEM-18} <u>Ec_20</u> (<i>bla</i> _{CTX-M-15}) 20071 (Madec JY et al., 2012) (<i>bla</i> _{CTX-M-15}), <i>bla</i> _{TEM-1C} <u>Ec_16</u> (<i>bla</i> _{CTX-M-15}) <u>27732</u> (Lupo A et al., 2018b) (<i>bla</i> _{CTX-M-55}) <u>Ec_42</u> (<i>bla</i> _{CTX-M-55}) <u>Ec_20</u> (<i>bla</i> _{CTX-M-15})	<u>Ec_61</u> : <i>bla</i> _{CTX-M-1} <u>27259</u> (Lupo A et al., 2018a): <i>bla</i> _{CTX-M-1} <u>27732</u> (Lupo A et al., 2018b): <i>bla</i> _{CTX-M-55} <u>Ec_42</u> : <i>bla</i> _{CTX-M-55}
<i>bla</i> _{CTX-M-1ep-725G} AY044436.1	F: AGCGAGCATTAGGCTGG ^d R: CAGCGCGCAGGATCT ^d MGB-TGGCTATGGCACCACCA-FAM	84	<u>Ec_61</u> (<i>bla</i> _{CTX-M-1}) <u>27259</u> (Lupo A et al., 2018a): <i>bla</i> _{CTX-M-1} <u>32137</u> (Lupo A et al., 2018a): <i>bla</i> _{CTX-M-1} <u>24424</u> (Dahmen S et al., 2013): <i>bla</i> _{CTX-M-3} , <i>bla</i> _{TEM-18}	

(continued)

Table 2

Primes and probes (TaqMan, Applied Biosystem) sequences of nine qPCRs on aminoglycosides resistance genes and features of positive (n = 32) and negative control isolates (n = 9). Genetic content of the isolates was determined by PCR or genome sequencing (underlined isolates) and analysis with ResFinder 3.2.

Gene, Accession number	Primer F; R; probe sequence (5'→3')	Amplicon size (bp)	Isolate containing the target and other genes relative to aminoglycosides resistance	Isolates target-free and other genes relative to aminoglycosides resistance ^a
<i>aac(3)-IIa</i> , AY333434.1	F: AGGACATCGTGACGTTCCGG R: AACCTGAAGGCTCGCAAGAG QSY-CGGAGTGGTTCGAAATGCTTCTCAAG-ABY	105	<u>Ec_37</u> 32189 (Lupo A et al., 2018b), <u>rmtB</u> , <u>aac(6)-Ib</u> <u>Ec_44</u> , <u>strA/B</u> , <u>aph(3)-Ia</u> , <u>ant(2)-Ia</u> <u>Kp_98</u> , <u>strA/B</u> , <u>aac(6)-Ib-cr</u> <u>Ab_94</u> , <u>ant(3)-IIa</u> , <u>aac(6)-Ian</u>	<u>Ec_12</u> : none <u>Ec_13</u> : none <u>Ec_55</u> : none <u>Ec_68</u> : none
<i>aac(3)-IV</i> , X01385.1	F: ATCGGTCAGCTTCTCAACT R: TCGTCCAGACTGACCAC QSY-TCCACAGCTCTTCCGTAGCGTCC-ABY	173	<u>Ec_01</u> <u>Ec_80</u> <u>Ec_84</u> , <u>strA/B</u> , <u>aph(3)-Ia</u> , <u>aph(4)-Ia</u> , <u>ant(3)-Ia</u> <u>Kp_103</u> , <u>ant(3)-Ia</u> <u>Ec_54</u> , <u>aac(3)-IIIa</u> , <u>aph(4)-Ia</u> , <u>aph(3)-Ia</u> , <u>ant(3)-Ia</u>	
<i>aac(6)-Ib</i> , JQ808129.1	F: AACTGCGAGCGATCCGA R: TGGCGTGTGTAACCATGTAC QSY-TACCTTGCTTCTCAAACCCCGCTTCTC-JUN	101	20071 (Madec JY et al., 2012), <u>aac(6)-Ib-cr</u> , <u>aac(3)-IIa</u> , <u>ant(3)-Ia</u> , <u>ant(2)-Ia</u> <u>Ec_04</u> , <u>aac(6)-Ib-cr</u> <u>Kp_102</u> , <u>strA/B</u> , <u>aac(6)-Ib-cr</u> , <u>ant(2)-Ia</u> 33904 (Lupo A et al., 2018b), <u>aac(6)-Ib</u> 32189 (Lupo A et al., 2018b), <u>aac(6)-Ib</u> , <u>rmtB</u>	
<i>strA</i> , M95402.1	F: TCAATCCCGACTCTTACCGG (Walsh F et al., 2011) R: CACCATGGCAACACCATATC MGB-TGGCTCGTGCAGAAC-VIC	126	34972 (Lupo A et al., 2017), <u>strA/B</u> , <u>aac(3)-IIa</u> , <u>aac(6)-Ian</u> 38208 (Lupo A et al., 2017) <u>strA/B</u> , <u>aac(3)-IIa</u> , <u>aac(6)-Ian</u> <u>Ab_107</u> , <u>strA/B</u> , <u>aac(3)-IIa</u> <u>Ab_109</u> , <u>strA/B</u> <u>aac(3)-IIa</u> , <u>aac(6)-Ian</u> , <u>ant(2)-Ia</u> <u>Ab_108</u> , <u>strA/B</u> <u>aac(3)-IIa</u> , <u>aac(6)-Ian</u> , <u>ant(2)-Ia</u>	
<i>strB</i> , M95402.1	F: ATCGCTTTGAGCTTTGTTTC (Walsh F et al., 2011) R: TCCGAGGCATT GCTCATCATT QSY-CGTCCAGCGGGGATTATAGCC-JUN	112	34972 (Lupo A et al., 2017), <u>strA/B</u> , <u>aac(3)-IIa</u> , <u>aac(6)-Ian</u> 38208 (Lupo A et al., 2017) <u>strA/B</u> , <u>aac(3)-IIa</u> , <u>aac(6)-Ian</u> <u>Ab_107</u> , <u>strA/B</u> , <u>aac(3)-IIa</u> <u>Ab_109</u> , <u>strA/B</u> <u>aac(3)-IIa</u> , <u>aac(6)-Ian</u> , <u>ant(2)-Ia</u> <u>Ab_108</u> , <u>aac(3)-IIa</u> , <u>aac(6)-Ian</u> , <u>ant(2)-Ia</u>	
<i>aph(3)-Ia</i> , V00359.1	F: ACCGAGGACAGTCCATAGGA R: ACCTTATTTTGGAGGAGGGAAA QSY-ATCTGGTATCGGCTCGGATTCCGA-JUN	104	<u>Ab_92</u> , <u>aac(3)-Ia</u> , <u>ant(2)-Ia</u> , <u>ant(3)-Ia</u> <u>Ab_93</u> , <u>aac(3)-Ia</u> , <u>ant(3)-Ia</u> <u>Ec_85</u> , <u>strA/B</u> , <u>ant(3)-Ia</u> <u>Ec_87</u> , <u>aac(6)-Ib-cr</u> <u>Ec_77</u> , <u>strA/B</u> , <u>aac(3)-IIa</u> 20071 (Madec JY et al., 2012), <u>aac(3)-IIa</u> , <u>ant(3)-Ia</u>	
<i>ant(2)-Ia</i> , EU247928.1	F: CGCGAGCTCGAGGC R: AITTCATACGCTTCGTCTGCC QSY-TCATGGAGGAGTGGACTATGGATTCTTAGCG-ABY	135	<u>Ec_19</u> , <u>strA/B</u> , <u>aph(3)-Ia</u> , <u>ant(3)-Ia</u> <u>Ab_109</u> , <u>strA/B</u> , <u>aac(3)-IIa</u> , <u>aac(6)-Ian</u> <u>Ec_79</u> , <u>strA/B</u> , <u>ant(3)-Ia</u> <u>Ec_89</u> , <u>strA/B</u> , <u>ant(3)-Ia</u> <u>Ec_17</u> , <u>aph(3)-Ia</u>	
<i>ant(3)-Ia</i> , AF313472.2	F: ACGTGTCCCGCATTTGG R: TCCTCGGCGGATTTT MGB-ACAGCCGACGTAACC-FAM	53	<u>Ec_64</u> , <u>strA/B</u> , <u>aph(3)-Ia</u> , <u>ant(2)-Ia</u> <u>Ec_25</u> , <u>aac(3)-IIa</u> <u>Ab_92</u> , <u>aph(3)-Ia</u> , <u>aac(3)-Ia</u> <u>Ab_93</u> , <u>aph(3)-Ia</u> , <u>aac(3)-Ia</u> <u>Ec_59</u>	
<i>rmtB</i> , AB103506.1	F: CTCACCTCCATCCTGGCCT R: TTATGCCGCCCATTC QSY-ACCGGCCCTTTGCCCG-JUN	89	32189 (Lupo A et al., 2018b) <u>aac(6)-Ib</u> , <u>aac(3)-IIa</u> 34248 (Lupo A et al., 2018b) <u>aac(6)-Ib</u>	<u>Ec_50</u> : <u>strA/B</u> , <u>aac(3)-IIIa</u> , <u>aph(3)-Ia</u> , <u>ant(3)-Ia</u> <u>Kt_105</u> : <u>strA/B</u> , <u>aac(3)-IIIa</u> , <u>aac(6)-Ib-cr</u> , <u>aph(3)-Ia</u> <u>Ec_43</u> : <u>strA/B</u> , <u>aac(3)-IIIa</u> , <u>ant(3)-Ia</u> <u>Ab_90</u> : <u>strA/B</u> , <u>aac(3)-IIa</u>

Ec = *Escherichia coli*; Ab = *Acinetobacter baumannii*; Kt = *Klebsiella terrigena*; Kp = *Klebsiella pneumoniae*.

^a All qPCRs were tested using the target-free *E. coli* CIP 74.26.

Table 3

Primes and probes (TaqMan, Applied Biosystem) sequences of qPCRs on fluoroquinolones resistance genes (*qnrB1*, *qnrS*, *aac(6)-Ib-cr*), colistin (*mcr-1*, *mcr-3*), inhibitors of folic acid pathways (*sul1*, *sul2*, *sul3*, *dhfrA1*, *dhfrA12*, *dhfrA17*), tetracyclines (*tetA*, *tetB*, *tetM*), and integrons (*int1* and *int2*) and features of positive and negative control isolates. Genetic content of the isolates was determined by PCR or genome sequencing (underlined isolates) and analysis with ResFinder 3.2.

Gene, Accession number	Primer F; R; probe sequence (5'→3')	Amplicon size (bp)	Isolate containing the target and other genes relative to the resistance conferred by target	Isolates target-free and other genes conten ^a
<i>qnrB1</i> , DQ351241.1	F: GTAGCGCATATACAGAAATACCAATC (Dhelly A et al., 2012) R: ATCTGAACCACTGAACGTCGC QSY-AAGTCGTGTGGAAAAGTGTGAGCTGTGG-JUN	131	<u>Es_106, <i>aac(6)-Ib-cr</i></u> <u>Ec_75, <i>oqxAR, aac(6)-Ib-cr</i></u> <u>Kp_98,</u> <u><i>oqxAR, aac(6)-Ib-cr</i></u> <u>Kp_104, <i>oqxAR, aac(6)-Ib-cr</i></u> <u>Kp_102, <i>oqxAR, aac(6)-Ib-cr</i></u> <u>Ec_84</u>	<u>Ec_12: none</u> <u>Ec_13: none</u> <u>Ec_55: none</u> <u>Ec_68: none</u>
<i>qnrS</i> , AB187515.1	F: CGAGCWGCTAACTTGGGTGA (Brown-Jaque M et al., 2018) R: GGCAATGTTGGAAACTTGCA (Brown-Jaque M et al., 2018) QSY-TCATTGAACAGGGTGATATCGAAGGCTGC-JUN	118	<u>34228 (Lupo A et al., 2018b),</u> <u><i>oqxAR, qnrS, aac(6)-Ib-cr</i></u> <u>Ec_85</u> <u>Kt_105, <i>oqxAR, qnrB, aac(6)-Ib-cr</i></u> <u>Ec_33</u>	
<i>aac(6)-Ib-cr</i> , GQ438247.1	F: AACTTGGCAGCGATCCGA R: TGGCGTGTGGAACCATGTAC MGB-TACGGTACCTGCCTCT-VIC	101	<u>Ec_26</u> <u>Ec_37</u> 20071 (Madec JY et al., 2012) 22611 (Dahmen S et al., 2013) <u>Kp_99, <i>oqxAR, qnrB4</i></u>	<u>Ec_17: <i>aac(6)-Ib</i></u> <u>32189 (Lupo A et al., 2018b): <i>aac(6)-Ib</i></u> <u>34248 (Lupo A et al., 2018b): <i>aac(6)-Ib</i></u> <u>33058 (Lupo A et al., 2018b): <i>aac(6)-Ib</i></u>
<i>mcr-1</i> , KP347127.1	F: TCGGTCAGTCCGTTGTCTT R: TGGTATCAGTAGATCCGG QSY-TCTTGACCCGACCCCAATCT-JUN	166	<u>Ec_05</u> <u>Ec_10</u> <u>30271 (Lupo A et al., 2018b)</u> <u>Ec_69</u> <u>Ec_43, <i>mcr3</i></u>	<u>Es_106</u> <u>27841 (Haenni M et al., 2018): <i>mcr-3</i></u> <u>27845 (Lupo A et al., 2018b): <i>mcr-3</i></u> <u>32524 (Haenni M et al., 2018): <i>mcr-3</i></u>
<i>mcr-3</i> , KY924928.1	F: CCAGTTGATGTTTCTGGTCG R: ATCATTAAAGGAGATCACGCCAC QSY-TCTCGATGAATGGCTATGAGAAAGACACCA-ABY	122	<u>27841 (Haenni M et al., 2018)</u> <u>32524 (Haenni M et al., 2018)</u> <u>33058 (Lupo A et al., 2018b)</u> <u>33904 (Lupo A et al., 2018b)</u> <u>Ec_43, <i>mcr1</i></u>	<u>Es_106</u> <u>32189 (Lupo A et al., 2018b)</u> <u>Ec_07</u> <u>Ec_69: <i>mcr-1</i></u>
<i>sul1</i> , X12868.1	F: CCGTGGCCTTCTGTAAAG (Czekalski N et al., 2012) R: TTCCGATCCGCTGAAGT (Czekalski N et al., 2012) QSY-CAGCGAGCCTTCCGGCGG-ABY (Czekalski N et al., 2012)	67	<u>Ec_26, <i>sul2</i></u> <u>Ec_37, <i>sul2</i></u> <u>Ec_48, <i>sul2</i></u> <u>Ec_43, <i>sul3</i></u> <u>Ab_92</u>	<u>Ec_57: <i>sul2</i></u> <u>Ec_18: <i>sul2</i></u> <u>Ec_41: <i>sul2</i></u> <u>Kp_105: <i>sul2</i></u>
<i>sul2</i> , M36657.1	F: CGGTGCGCTTCGATT (Czekalski N et al., 2012) R: CCGCGCAGAAAGGATT (Czekalski N et al., 2012) MGB-TGCTTCTGCTGTTTCG-FAM	60	<u>Ec_48, <i>sul1</i></u> <u>Ec_51</u> <u>Ab_90</u> <u>Ec_08, <i>sul3</i></u> <u>Ec_50, <i>sul1</i></u>	<u>Ec_26: <i>sul1</i></u> <u>Ec_04: <i>sul1</i></u> <u>Ec_17: <i>sul3</i></u> <u>Kp_97: <i>sul1</i></u>
<i>sul3</i> , AJ459418.2	F: TAGGCTGCAAGATAGGGAAATG R: CAACGCCACTTCAGTGTATC QSY-TGGAGCAGATGTGATTGTTGGAGC-JUN	268	<u>Ec_28</u> <u>Ec_39, <i>sul1, sul2</i></u> <u>Ec_46, <i>sul2, sul3</i></u> <u>Ec_56, <i>sul2, sul3</i></u>	<u>Ec_49: <i>sul1, sul2</i></u> <u>Ec_72: <i>sul2</i></u> <u>Ec_81: <i>sul2</i></u> <u>22611 (Dahmen S et al., 2013): <i>sul1, sul2</i></u>
<i>dhfrA1</i> , X00926.1	F: TCGAAGAATGGAGTTATCGGGA R: TGTGATGTAAACTGAACTGTGTTA QSY-TGGAGCATTACCAACCGAAAGTATGC-ABY	184	<u>Ec_08, <i>sul2</i></u> <u>Ec_38</u> <u>27259</u> <u>(Lupo A et al., 2018a), <i>dhfrA17</i></u> <u>Ec_06</u> <u>Ec_63</u> <u>Ab_91</u>	<u>Ec_72: <i>dhfrA5, dhfrA17</i></u> <u>22611 (Dahmen S et al., 2013): <i>dhfrA12</i></u> <u>Kp_105</u> <u>Kp_102: <i>dhfrA14</i></u>
<i>dhfrA12</i> , Z21672.1	F: TGCTGCGATGGAGCC R: GACGGTCCGTAGAGGCTTG QSY-TTCGAGACTCACTGAGGAAAAGTCTG-ABY	158	<u>Ec_17</u> <u>Ec_47</u> <u>Ec_08, <i>dhfrA17</i></u> <u>Ec_54</u> <u>45833 (Lupo A et al., 2018a)</u>	<u>37698 (Lupo A et al., 2018a): <i>dhfrA17</i></u> <u>Ab_94</u> <u>Ab_91: <i>dhfrA1</i></u> <u>Ab_95: <i>dhfrA1</i></u>
<i>dhfrA17</i> , AF169041.1	F: CGGTAATCGGTAGTGGTCTGAT R: TTCCGTTCTTGACACTACTGCAT QSY-TTGACTCTATGGGTGTTCTCCAAATCGCA-ABY	162	<u>Ec_18, <i>dhfrA1</i></u> <u>27259 (Lupo A et al., 2018a)</u> <u>Ec_08, <i>dhfrA12</i></u> <u>37698 (Lupo A et al., 2018a)</u> <u>Ec_35</u>	<u>Ab_94</u> <u>45833 (Lupo A et al., 2018a): <i>dhfrA12</i></u> <u>Ab_91: <i>dhfrA1</i></u> <u>Ab_95: <i>dhfrA1</i></u>
<i>tetA</i> , X00006.1	F: TCTGAGCACTGTCGGCTC R: ACAGGTCCGACGGCAA QSY-TGGTCACTCGAAGCAGTCACCG-JUN	156	<u>Ec_52</u> <u>Ec_06</u> <u>Ec_05, <i>tetM</i></u> <u>Es_106</u> <u>Kp_105</u>	<u>Ab_94: <i>tetB</i></u> <u>Ab_90: <i>tetB</i></u> <u>Ec_55: none</u> <u>Ec_68: none</u>
	F: TGTGGCAGGAAGAATAGCCAC	221	<u>34972 (Lupo A et al., 2017)</u>	<u>Ec_13: none</u>

(continued)

Table 3 (Continued)

Gene, Accession number	Primer F; R; probe sequence (5'→3')	Amplicon size (bp)	Isolate containing the target and other genes relative to the resistance conferred by target	Isolates target-free and other genes conten ^a
<i>tetB</i> , EU252517.1	R: ATCCCTGTAAG CACCTTGCTG QSY-TTGGCTGGTGGGATCGCTT-ABY		Ab_90 Ab_107 Ab_92, <i>tetA</i> Ab_91	Ec_55: none Ec_68: none Ab_92: <i>tetA</i>
<i>tetM</i> , X04388.1	F: CGGTTCTCTGGACTTAAATCAATC (Peak N et al., 2007) R: AGTCGTCACATCCACCATAC QSY-TGTTACAGCCATAGCGTATCCCTCC-JUN	102	Ec_39, <i>tetA/B</i> Ec_51, <i>tetA</i> Ec_05, <i>tetA</i> Ec_64, <i>tetB</i> Ec_24	Ec_40: <i>tetA/B</i> Ec_73: <i>tetB</i> Ec_55: none Ec_68: none
<i>int1</i> , EF078697.1	F-GCCTTGATGTTACCCGAGAGC (Barraud O et al., 2010) R-GATCGGTGCAATGCGTGTG (Barraud O et al., 2010) QSY-ATTCCTGCCGCTGTTCTGGGTTT-ABY (Barraud O et al., 2010)	196	Ec_38, <i>int2</i> Kp_105 27259 (Lupo A et al., 2018a) 32137 (Lupo A et al., 2018a) Ec_85	Ec_12: none Ec_13: none Ec_55: none Ec_68: none
<i>int2</i> , AJ001816.1	F-GACCGCTACCCTCTGTTATCTCTG (Barraud O et al., 2010) R-GCTTTCCACCCTTACCGT (Barraud O et al., 2010) QSY-CGTCGTGTATGGTGAGGTTGC-JUN (Barraud O et al., 2010)	194	Ec_38 Ec_51 Ec_18 Kp_105 Ec_07	

Ec = *Escherichia coli*; Ab = *Acinetobacter baumannii*; Kt = *Klebsiella terrigena*; Kp = *Klebsiella pneumoniae*; Es = *Enterobacter sakazakii*.

^a All the qPCRs were tested using the target-free *E. coli* CIP 74.26.

of target DNA generating values in the linear range of the reaction, with a coefficient variation <25% (Foorootan et al., 2017).

2.4. Diagnostic sensibility and specificity

Diagnostic sensitivity and specificity were estimated using bacterial isolates available in the laboratory collection. The presence or absence of the target in the positive controls, at least five each except for the *rmtB* gene ($n=3$) and the *bla*_{OXA-181} gene ($n=2$), was validated by PCR or next-generation sequencing (NGS) of the genome and ARDs analysis (ResFinder 3.2.0).

3. Results and discussion

3.1. General features of the qPCR assays

Forty-four qPCRs with common amplification conditions were developed in this study: 42 on the most common ARDs in *Enterobacteriales*, 2 on *intI* integrons genes, and one on the 16S rDNA gene for bacteria quantification. Six reactions had 100% efficiency, whereas the remaining ones had acceptable values (110% <E> 95%). LoQ varied between one and 5.7×10^3 gene copies number (Fig. 1). By testing at least five isolates carrying the intended target and five target-free isolates, all qPCRs demonstrated 100% diagnostic sensitivity and specificity considering that all positive control DNAs generated a positive result unlike negative controls, confirmed by previously developed PCR or NGS.

3.2. qPCR on the 16S rRNA encoding gene

Quantification of the ARDs relatively to bacterial abundance is necessary when analyzing the action of disruptors on bacterial communities, such as antibiotics on the intestinal microbiota. To do so, one qPCR targeted a conserved part of the 16S rDNA. A previously published system was adapted reducing the size of the primers to adapt the Tm (Nadkarni et al., 2002) (primers: F-TCTACGGGAGGCAGCA; R-ACTACCAGGGTATCTAATCCTGTT; probe: QSY-CGTATTACCGCGCTGCTGGCAC-JUN). The assay was optimized using a PCR product generated with the DNA of *E. coli* CIP 74.26 (Fig. 1a), as representative of the most abundant culturable species of mammalian intestinal tract (Conway et al., 2004),

and tested on further 13 species (*Acinetobacter baumannii* 32865, *Proteus mirabilis* NKU, *Enterococcus casseliflavus* CIP 102598, *Enterococcus faecalis* CIP 103214, *Enterococcus faecium* CIP 103226, *Enterococcus gallinarum* CIP 103013, *Klebsiella pneumoniae* CIP 106769, *Streptococcus agalactiae* A909, *Staphylococcus aureus* CIP 7625, *Staphylococcus epidermidis* CIP 106299, *Staphylococcus haemolyticus* CIP 107204, *Staphylococcus pseudintermedius* CIP 108864, *Streptococcus uberis* CIP 103219), all generating a positive amplification.

3.3. qPCRs on beta-lactam resistance genes

Beta-lactams are largely used in human and veterinary medicine (ECDC, 2019; ESVAC, 2019), because of their large spectrum of action and a favorable pharmacodynamics (Turck, 1982). Their enzymatic degradation by beta-lactamases has emerged and disseminated dramatically (Seiffert et al., 2013). Here, 17 qPCRs were designed on beta-lactam resistance genes.

Penicillins are important in veterinary medicine (Bourély et al., 2019) but the *bla*_{TEM} and *bla*_{SHV} are the most worldwide diffused ARDs (Alonso et al., 2017; Clockaert et al., 2007; Liakopoulos et al., 2018; Partridge et al., 2005; Zankari E et al., 2012). The *in silico* analysis of previously published qPCRs demonstrated elevated primers Tm (Alfaresi and Elkoush, 2010; Lachmayr et al., 2009; Roschanski et al., 2014), dimerization in primers (Lachmayr et al., 2009; Roschanski et al., 2014), or too many G/C in the probe's 3'-end (Alfaresi and Elkoush, 2010), thus qPCRs were designed *de novo* on *bla*_{TEM} and *bla*_{SHV} genes (Fig. 1b). The *bla*_{TEM-52} and *bla*_{SHV-5-12} alleles encoding ESBLs (Nüesch-Inderbinen et al., 1997; Poyart et al., 1998) were detected (Table 1), however, penicillinase and ESBL variants could not be discriminated due to the complexity and variation of the nucleotide sequence in the region pertinent to the discrimination. A previous study discriminated two SNPs in position 164 and 240 for *bla*_{TEM} ESBL (Mroczkowska et al., 2008). However, this did not include other crucial SNPs of *bla*_{TEM-52} (Bradford, 2001). This intrinsic limitation of the methodology could affect the epidemiological data and infection control, thus other approaches are necessary to distinguish ESBL and penicillinase encoding alleles.

The *bla*_{CTX-M5} are nowadays the most globally expanded genes coding for ESBLs. They include four main groups, *bla*_{CTX-M-1-gp}, *bla*_{CTX-M-2-gp}, *bla*_{CTX-M-8-gp}, and *bla*_{CTX-M-9-gp}, with several alleles varying in

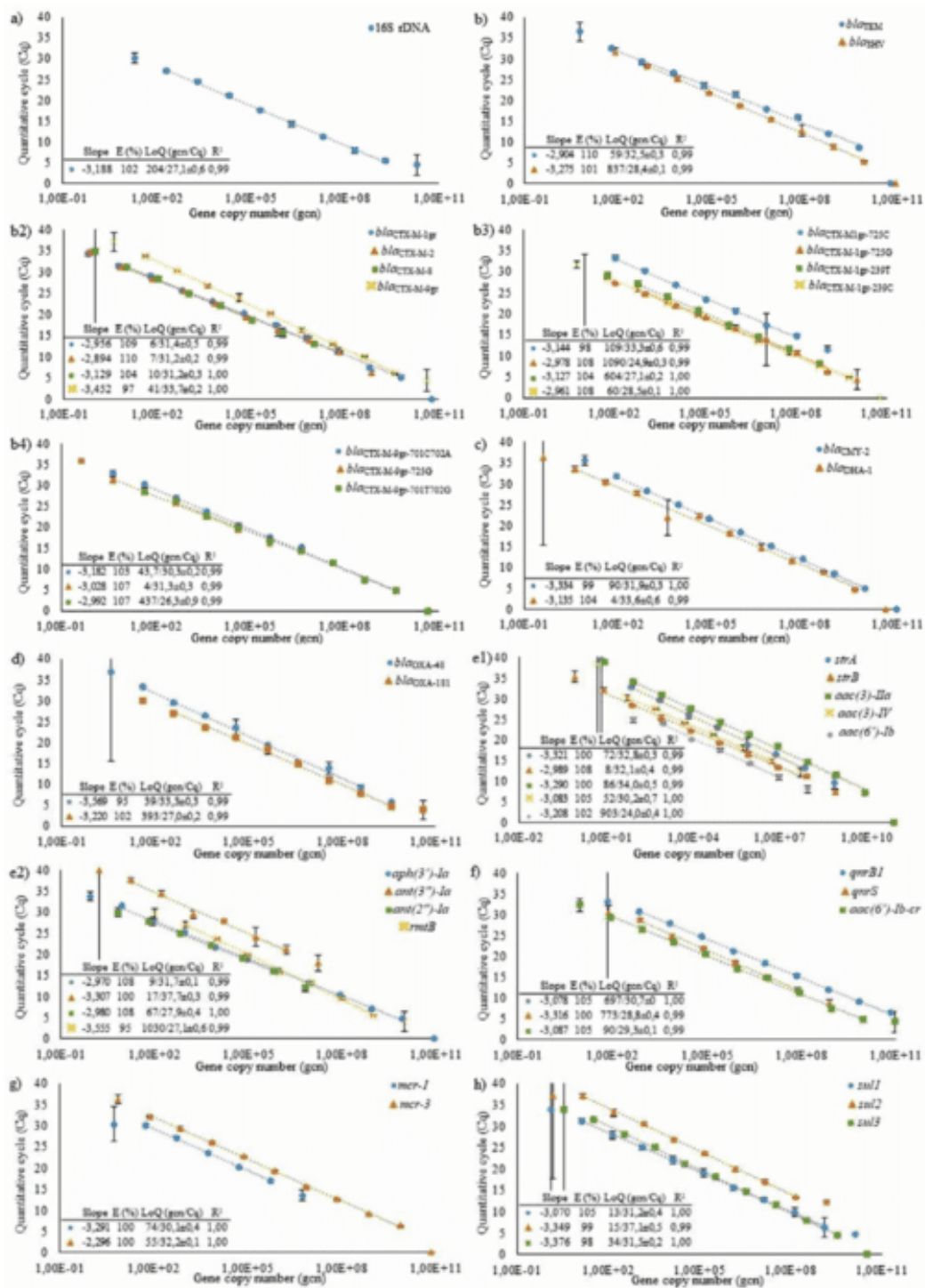


Fig. 1. Features of qPCRs using hydrolysis probes (TaqMan, Applied Biosystem) on the most common acquired antibiotic resistance genes in Enterobacteriales in Europe, 16S rDNA, and *int1* and *int2* genes encoding integrases of class 1 and 2 integrons. Standard curves were obtained using serial (10-fold) dilutions of purified PCR products in technical triplicates. Equations were obtained from the mean quantitative cycle (Cq) values plotted against the gene copies number (gcn). E = efficiency; LoQ = limit of quantification.

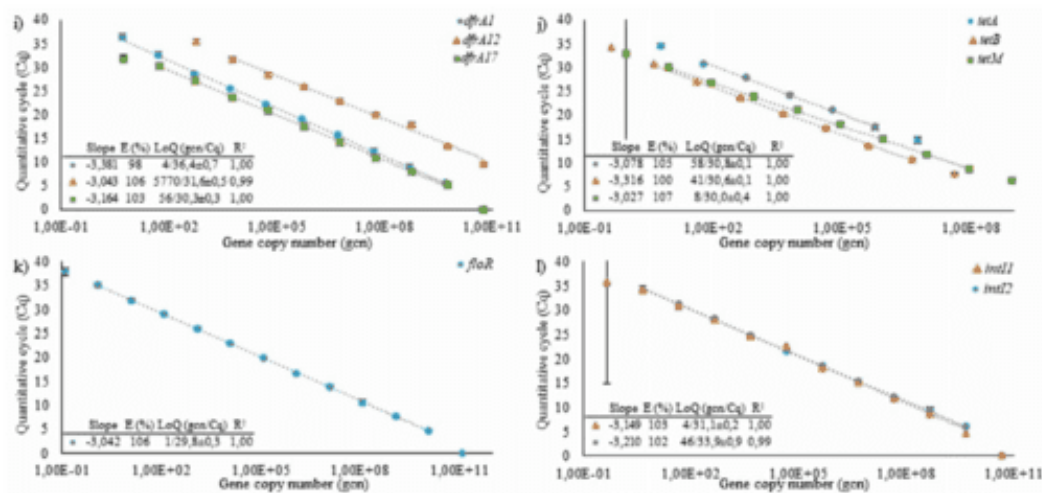


Fig. 1. Continued.

prevalence according to the host and the geographical origin (Livermore et al., 2007). The association of these genes with insertion sequences and the location on a wide family of conjugative plasmids has contributed to their success (Carattoli, 2013). The peculiar geographical and host distribution of *bla*_{CTX-Ms} provides a useful epidemiological indicator to understand the evolution of ARDs dissemination. qPCRs on *bla*_{CTX-Ms} exist already, but in most of the cases the discrimination remained at the group level (Birkett et al., 2007; Mangold et al., 2013; Roschanski et al., 2014; Sittová et al., 2015) or focused on the detection of the *bla*_{CTX-M-1-*gr*} members (Calero-Cáceres et al., 2014; Quirós et al., 2014). In this study the qPCRs allowed two levels of discrimination: four qPCRs discriminated the *bla*_{CTX-M} groups (Table 1) all with acceptable efficiency (Fig. 1, b2); seven assays discriminated the variants *bla*_{CTX-M-1-3-15-55} of the *bla*_{CTX-M-1-*gr*} and *bla*_{CTX-M-9-14-27} of the *bla*_{CTX-M-9-*gr*}. For the *bla*_{CTX-M-1-*gr*} two SNPs were discriminative one at position 239 (*bla*_{CTX-M-1-55} presented a thymine, whereas *bla*_{CTX-M-3-15} presented a cytosine) and one at position 725 (*bla*_{CTX-M-1-3} presented an adenine, whereas *bla*_{CTX-M-15-55} had a guanine) (Fig. S1, a). Similarly, *bla*_{CTX-M-9-14-27} presented three discriminative residues at position 701, 702, and 725 (*bla*_{CTX-M-9}: 701C702A, 725A; *bla*_{CTX-M-14}: 701T702G, 725A; *bla*_{CTX-M-27}: 701T702G, 725G) (Fig. S1, b). The probe designed around the 725 residue produced amplification exclusively on the 725G variant allowing *bla*_{CTX-M-27} discrimination. For each SNP a probe with a MGB quencher and a different fluorophore was designed and used in the same well with a unique couple of primers (Table 1). The probes enter in competition for the proper variant and the fluorophore emission corresponding to the probe with 100% nucleotide match to the target emits a stronger signal that can be interpreted with the genotyping algorithm. The assays were evaluated using the mixed probes and opportune targets (Fig. 1, b3-b4) and were 100% discriminative (Fig. 2), as confirmed by PCR (Lupo et al., 2018b; Shibata et al., 2006) or NGS on 64 positive and 41 negative controls, respectively (Table 1).

Among class C beta-lactamases, *bla*_{CMY-2} is the most common followed by *bla*_{DHA-1} (Guo et al., 2014; Hong et al., 2019; Martin et al., 2011; Mo et al., 2019; Pepin-Puget et al., 2020; van den Bunt et al., 2020).

For the *bla*_{CMY-2} qPCR primers previously published were adopted (Roschanski et al., 2014). For the *bla*_{DHA-1} qPCR the primers were modified from Swayne et al., 2013 to rise the Tm and degenerating one nucleotide to amplify all variants. The probes were designed *de novo*

and qPCRs validated on *bla*_{CMY-2} and *bla*_{DHA-1} (Fig. 1, c). Specificity and sensitivity were validated by NGS (n=16) and PCR (n=1) (Table 1) (Carattoli et al., 2002).

Those *bla*_{OXA} genes encoding class D beta-lactamases with carbapenemase activity represent a great health problem due to their association with mobile elements facilitating the dissemination and for the nonsusceptibility to common beta-lactamase inhibitors (Walther-Rasmussen et al., 2006). Their detection by molecular methods is of particular interest, considering that in certain circumstances they confer low level resistance to carbapenems rendering their phenotypic detection challenging (Thomson KS, 2010).

Previous qPCRs on *bla*_{OXA} genes have been reported (Brown-Jaque et al., 2018; Chavda et al., 2016; Huang et al., 2012; Swayne et al., 2011). Here, MGB quencher was used for the detection of *bla*_{OXA-48} and *bla*_{OXA-181} to achieve a better specificity allowing discrimination of *bla*_{OXA-48} from the *bla*_{OXA-181} variant (Table 1, Fig. 1, d).

3.4. qPCRs on aminoglycosides resistance genes

Besides their toxicity, and the widely disseminated resistance by enzymatic modifications, aminoglycosides continue to be largely used in animals and humans. Often, a single isolate harbors several aminoglycosides resistance genes located on diverse mobile genetic elements (Bliziotis et al., 2005; Gow et al., 2008; Ho P-L et al., 2010; Ramirez et al., 2010; Sandvang et al., 2000; Shaw et al., 1993; Sundin et al., 1996; Vakulenko et al., 2003; van Duijkeren et al., 2019). In this study, nine qPCRs were developed to detect aminoglycosides resistance genes (Fig. 1, e1-e2; Table 2). For the *strA-strB* qPCRs the assay of Walsh F et al., 2011 was adopted modifying the primer sequences to avoid dimerization and using last-generation fluorophores and quenchers for the probe (Table 2), whereas the *ant(3'')-Ia* gene qPCR was designed *de novo* as the previously designed probe had a too low Tm (Walsh et al., 2011). Also the *rmtB* gene qPCR was designed *de novo* to adapt the experimental conditions to the other targets (Guo et al., 2014).

The isolates used as positive controls contained other ARDs validated by PCR or NGS (Fritsche et al., 2008; Lupo et al., 2017; Lupo et al., 2018b; Madec et al., 2012; Park et al., 2006) (Table 2).

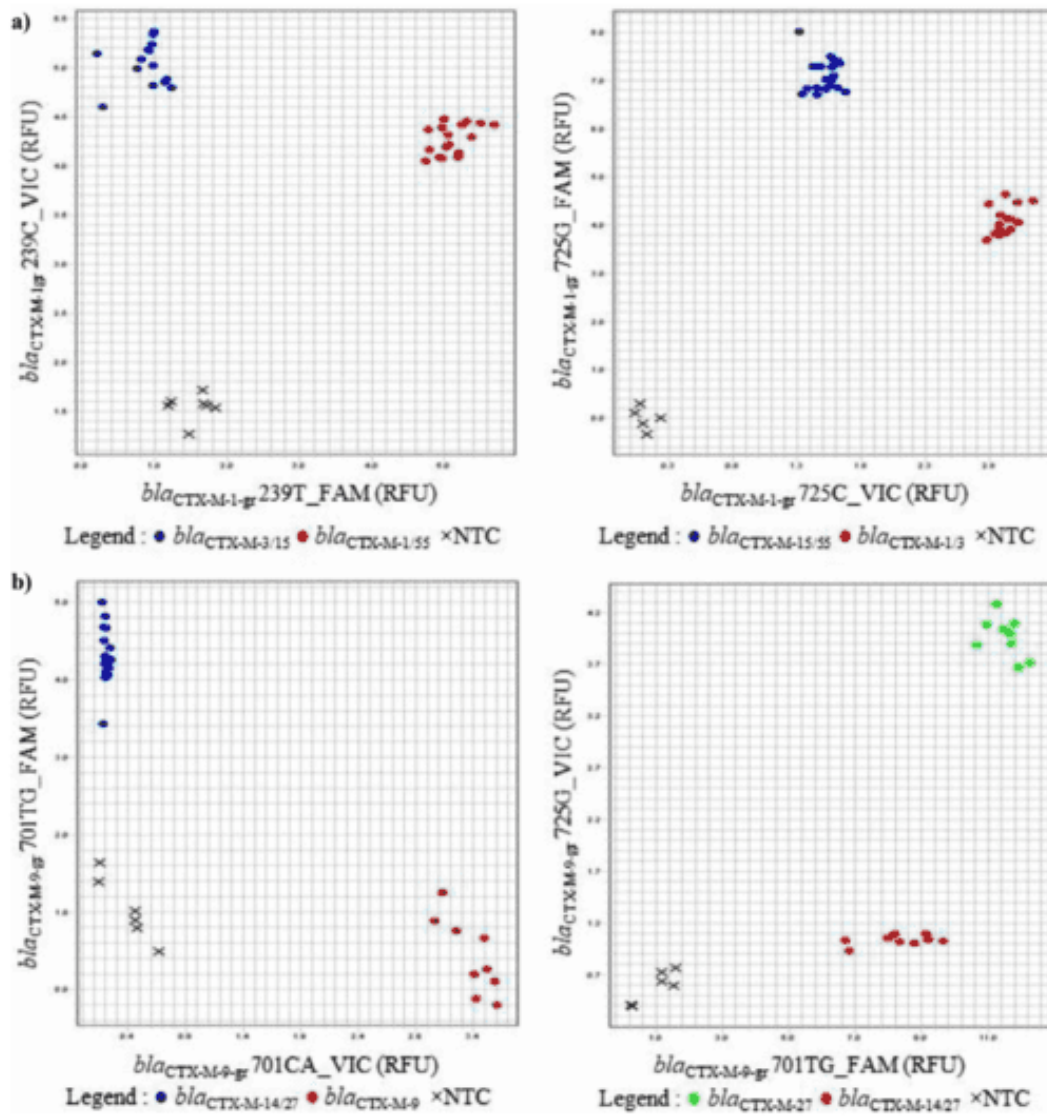


Fig. 2. Cluster plot of single nucleotide polymorphism (SNP) genotyping using hydrolysis probes (TaqMan, Applied Biosystem) on a) *bla*_{CTX-M-15/55} and b) *bla*_{CTX-M-9} most common variants. RFU: relative fluorescence unit. a) The allele 239C, whose probe is VIC labeled, is relative to *bla*_{CTX-M-15/55}, whereas the 239T allele, whose probe is marked with FAM, is relative to *bla*_{CTX-M-15/55}. The allele 725G, whose probe is labeled with FAM, is relative to *bla*_{CTX-M-15/55}, whereas the allele 725C, whose probe is marked with VIC, is relative to *bla*_{CTX-M-15/55}. b) The allele 701C/702A, whose probe is labeled with VIC, is relative to *bla*_{CTX-M-9}. The allele 701T/702G, whose probe is labeled with FAM, is relative to *bla*_{CTX-M-9}. Only isolates harboring the 725G allele whose probe is labeled with VIC and relative to *bla*_{CTX-M-27} produced an amplification.

3.5. qPCRs on fluoroquinolones resistance genes

Three qPCRs were conceived for detecting acquired fluoroquinolones resistance genes. For the *qnrS* gene the forward primer and a modified reverse primer, to obtain a proper T_m , and a modified probe, in order to avoid hybridization with the primers, were adapted from Colomer-Lluch et al., (2014). For the *qnrB1* gene qPCR the assay designed by Dheilly et al., (2012) was modified to adapt the T_m of the forward primer and avoid dimer formation for the reverse primer, whereas the probe was designed *de novo*. The *aac(6)-Ib-cr* gene qPCR was designed *de novo* and it amplifies the *aac(6)-Ib-cr* variant, exclusively, allowing discrimination from *aac(6)-Ib*. The presence or absence of the target was proved by PCR or NGS (Dahmen et al.,

2013; Lupu et al., 2018a; Lupu et al., 2018b; Madec et al., 2012; Park et al., 2006; Robicsek et al., 2006) (Table 3, Fig. 1, f).

3.6. qPCRs on colistin resistance genes

qPCRs on the *mcr1* gene have been developed, previously (Chabou et al., 2016; Daniels et al., 2019). Here, the design from Chabou et al., (2016) was modified to avoid dimers formation of the primers and using a part of the probe, avoiding the guanine at the 3'-end. A qPCR assay was developed *de novo* for the *mcr-3* allele. All the isolates confirming to harbor the gene by PCR (Yin W et al., 2017) or NGS produced a positive result (Table 3). The negative controls did not produce amplification although some harbored a *mcr* gene

different from the intended target (Haenni et al., 2018; Lupo et al., 2018b). The qPCRs were analytically highly sensitive (Fig. 1, g).

3.7. qPCRs on folic acid synthesis inhibitor resistance genes

For the *sulI* gene the primers designed by Brown-Jaques et al. (Brown-Jaque et al., 2018) were used, whereas the probe was designed *de novo* similarly to the *sul2/3* alleles. The reactions had similar efficiency and analytical sensitivity (Fig. 1, h). All positive controls produced an amplification, unlike negative controls although they contained other *sul* alleles than the intended one (Dahmen et al., 2013; Di Conza J et al., 2002; Lupo et al., 2017; Nigro et al., 2012) (Table 3).

Among *dfr* genes only the group *dfrA* is also detected in animal isolates (Poirel et al., 2018) and *dfrA1/12/17* alleles are the most diffused, located on class 1 and 2 integrons (Piccirillo et al., 2014; Sköld O 2001).

No previous qPCR assays have been designed for these genes. The novel qPCRs had variable analytical sensitivity and efficiency (Fig. 1, i) and were diagnostically specific (Table 3).

3.8. qPCRs on tetracyclines resistance genes

Previous design on *tetA*, *tetB* and *tetM* have been published (Guaradon M et al., 2011; Peak N et al., 2007; Walsh F et al., 2011), however due to dimers formation between primers and probes three *de novo* assays were designed, here. The analytical sensitivity was high for all the genes (Fig. 1, j). The assays were 100% diagnostically specific and sensitive as confirmed by PCR assays (Lupo A et al., 2017) (Table 3).

3.9. qPCRs on phenicol resistance gene

The *floR* qPCR here developed (Accession no.: AF231986.2; primers: F: AGATCGGATTTCAGCTTTGCC; R: CAAAGGACTTCGCGAAACG; probe: QSY-CGCCACTGTCCGCTTGTAATGATC-JUN; amplicon size: 75 bases) was validated for analytical features (Fig. 1, k) and using five positive controls (20318, Ec39, 27841 (Haenni et al., 2018), 27845 (Haenni et al., 2018), 32189 (Lupo et al., 2018b)). Negative controls were the same than tetracycline resistance genes qPCRs (Table 3).

3.10. qPCR assays on *int1* and *int2* genes of class 1 and 2 integrons

Two *intI* alleles are principally reported from animal isolates, *intI1* and *intI2* determining integrons class 1 and 2 (Deng et al., 2015; Mazel, 2006).

For this study, primers were adapted from Barraud et al. (Barraud et al., 2010) for the detection of the *intI1* and *intI2* alleles in order to satisfy the Tm requirements and last generation fluorophores and quenchers were used for the probes (Table 3). The assay remained with acceptable efficiency compared to the previous design, where both assays had a 97% efficiency. The analytical sensitivity was slightly decreased for *intI1* and increased for *intI2* (Barraud et al., 2010) (Fig. 1, l).

4. Conclusion

This study provides the largest noncommercial inventory of qPCRs ($n = 44$) based on hydrolysis probes, operating with the same amplification conditions, including the 16 rDNA, allowing ARDs quantification with accuracy, relatively to the bacterial abundance, avoiding laborious calibration curves during experiments, as required using generic dyes. To ensure the accuracy of the design, 435 reactions of validation on positive and negative controls were performed and validated with alternative methods (PCR and genome sequencing). All

qPCRs had 100% diagnostic specificity, confirming the main advantage for using probes. Here, the first qPCR based genotyping is proposed to easily and rapidly differentiate the main epidemiologically relevant *bla*_{CTX-M5} variants in both humans and animals. The turn-around time from plate preparation to analysis of results was 2 hours for seven samples, for which purified DNA was available, making the system suited to both epidemiological or research purposes. The limit for diagnostic application is linked to the articulated ARDs combination that can occur in *Enterobacterales*, rendering difficult the phenotype prediction of pathogens. The *in silico* prediction of primers and probes performance corresponded to the real experimental conditions, suggesting that addition of other targets, following the same design, will not necessitate optimization efforts, which is the most expensive step related to qPCR based on probes. The *in silico* design took in account the possibility of multiplexing, putting the basis for further high-throughput developments.

Guidelines for reporting qPCR evaluation are available, however, the analytical features of previously published qPCRs are heterogeneously reported, thus a comparison among studies is challenging.

Overall, this study with this comprehensive, evolutive and robust qPCR inventory will contribute to the fight against ARDs and to investigations on ARDs changes in bacterial communities.

Funding support

This work and Tony Rochegüe thesis was supported by the LABEX ECOFECT (ANR-11-LABX-0048) of Université de Lyon, within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR) and proper funding from ANSES.

Declaration of competing interest

The authors report no conflicts of interest relevant to this article after Funding support.

Acknowledgments

Authors are grateful to all the research assistants of the AVB unit at the ANSES-Lyon.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.diagmicrobio.2021.115328.

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2. Impact of antibiotic therapies on resistance genes and composition of the intestinal microbiota of animals

The 44 qPCR assays were used to amplify and quantify ARGs in the stools of calves enrolled in the study. ARGs were quantified absolutely and relatively to the general bacterial amount, as estimated by the amplification of a conserved part of the 16S rDNA.

Concerning the *bla*_{TEM} gene, which could be directly selected by amoxicillin, treated calves (TC) and untreated calves (UC) presented an average comparable quantification at T0 (number of copies, Noc: 35.6 x 10³, TC; 42 x 10³, UC), thus before the amoxicillin treatment for the TC. Similarly, TC and UC contained a comparable amount of bacteria in their feces relatively to the amplification of a conserved part of the 16S encoding gene (Noc: 26.2 x 10⁵, TC; 31.3 x 10⁵, UC). The ratio *bla*_{TEM}/16S rDNA was 0.013 in both TC and UC groups. At the end of amoxicillin treatment the amount of *bla*_{TEM} gene increased in the TC group (Noc: from 35.6 x 10³ to 61.3 x 10³), whereas it decreased in the UC group (Noc: from 42 x 10³ to 15.6 x 10³). Bacterial population decreased in TC (Noc: from 26.2 x 10⁵ to 15.1 x 10⁵), whereas it remained constant in the UC group (Noc: from 31.3 x 10⁵ to 30.1x10⁵). In average, *bla*_{TEM}/16S rDNA ratio was eight-fold higher in TC (0.04) than UC (0.005). One week after the end of amoxicillin treatment, the UC group still presented a constant amount of bacteria (16S rDNA: 28 X 10⁵ noc) and a novel decrease, compared to T1, of the *bla*_{TEM} gene amount (Noc: from 15.6 x 10³ to 5.8 x 10³). A decline of the *bla*_{TEM} gene amount (Noc: from 61.3 x 10³ to 15.9 x 10³) was observed in the TC group, as well, at levels even lower to what was observed at T0. In TC group, bacterial amount remained similar to what was observed in T1 (16S encoding gene: 18.9 x 10⁵ noc). The *bla*_{TEM}/16S rDNA ratio decreased in both groups (TC: 0.008; UC: 0.002)

Some other ARGs followed the same dynamic of the *bla*_{TEM} gene, namely *tetA*, conferring resistance to tetracycline, *strA/B* conferring resistance to streptomycin, *aph(3')-Ib* conferring resistance to kanamycin, and *intI1* encoding the integrase of class 1 integrons.


These results suggested that amoxicillin treatment contributed to *bla*_{TEM} gene selection and co-selection of resistance to other classes of antibiotics. However, statistical analysis did not found a significant difference in the amount of *bla*_{TEM} at the three samplings and between treated and untreated calves. This lack of difference could be due to confounding factors. All treated calves received a varying amoxicillin regimen, probably at the base of a certain degree of variation observed between treated calves of the *bla*_{TEM} amount (mean Cq: 24.3 ± 4.6, T0;

23.6 ± 3.2, T1; 25.26 ± 4.1). Calves differed in ages, a factor that could influence amount of bacteria resident in the IM and of ARGs.

These results were published in a paper where the current longitudinal studies analyzing the effect of antibiotics on the IM composition and AMR in animals were reviewed. The review was published in the journal “Animals (Basel)” and has been assigned to section “Veterinary clinical studies” (<https://doi.org/10.3390/ani11113280>).

Review

Impact of Antibiotic Therapies on Resistance Genes Dynamic and Composition of the Animal Gut Microbiota

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Citation: Rochegüe, T.; Haenni, M.; Mondot, S.; Astruc, C.; Cazeau, G.; Ferry, T.; Madec, J.-Y.; Lupo, A. Impact of Antibiotic Therapies on Resistance Genes Dynamic and Composition of the Animal Gut Microbiota. *Animals* **2021**, *11*, 3280. <https://doi.org/10.3390/ani1113280>

Academic Editor: Eva Bussalleu

Received: 10 October 2021

Accepted: 11 November 2021

Published: 16 November 2021

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Simple Summary: Antibiotics perturb the gastrointestinal microbiota by killing bacteria beneficial for animal health and favoring the emergence of potential pathogens. Furthermore, antibiotics favor the emergence of resistant bacteria. Current knowledge on animals' intestinal microbiota and effects of antibiotics is blurred by the various posology, administration routes, and implemented methodologies for its analysis. We summarized 71 studies analyzing the administration of antibiotics by different routes, conducted on the main food-producing and companion animals, highlighting differences in the methodology applied for the intestinal microbiota and antibiotic resistance analysis. Overall, therapeutic dosage decreased bacterial species diversity and richness in the microbiota and selected antibiotic resistance genes. For non-therapeutic dosage, information on the selection of antibiotic resistance was scarce and the effect on the intestinal microbiota scattered. Understanding the gut microbiota composition and function in animals could open up strategies for its modulation to improve animal health and performance, and to minimize the negative impact of antibiotics.

Abstract: Antibiotics are major disruptors of the gastrointestinal microbiota, depleting bacterial species beneficial for the host health and favoring the emergence of potential pathogens. Furthermore, the intestine is a reactor of antibiotic resistance emergence, and the presence of antibiotics exacerbates the selection of resistant bacteria that can disseminate in the environment and propagate to further hosts. We reviewed studies analyzing the effect of antibiotics on the intestinal microbiota and antibiotic resistance conducted on animals, focusing on the main food-producing and companion animals. Irrespective of antibiotic classes and animal hosts, therapeutic dosage decreased species diversity and richness favoring the bloom of potential enteropathogens and the selection of antibiotic resistance. These negative effects of antibiotic therapies seem ineluctable but often were mitigated when an antibiotic was administered by parenteral route. Sub-therapeutic dosages caused the augmentation of taxa involved in sugar metabolism, suggesting a link with weight gain. This result should not be interpreted positively, considering that parallel information on antibiotic resistance selection was rarely reported and selection of antibiotic resistance is known to occur also at low antibiotic concentration. However, studies on the effect of antibiotics as growth promoters put the basis for understanding the gut microbiota composition and function in this situation. This knowledge could inspire alternative strategies to antibiotics, such as probiotics, for improving animal performance. This review encompasses the analysis of the main animal hosts and all antibiotic classes, and highlights the future challenges and gaps of knowledge that should be filled. Further studies are necessary for elucidating pharmacodynamics in animals in order to improve therapy duration,

standard approaches in microbiota research [22]. The first molecular approaches consisted in sequencing one of the hypervariable regions (V1–V9) of the 16S rRNA operon (rDNA) [23–25]. Nowadays, microbiome studies that relate to 16S rDNA gene approaches are mostly sequencing the near full length of the operon, combining short and long-reads sequencing [26,27]. In general, obtained and assembled sequences are assigned to the so-called “Operational Taxonomic Units” (OTUs); each of these will include sequences with 97% nucleotide identity with each other and, in turn, will show similarity to known 16S rDNA sequences. More recently, amplicon sequencing variants (ASV) or ribosomal sequencing variants (RSV) were implemented for microbiota analysis [28].

Parameters often used to describe GIM are richness, consisting in the absolute number of OTUs in each sample and diversity. Diversity intrinsic to the sample is referred to as alpha-diversity, often estimated by the Shannon index, whereas diversity between samples is addressed as beta-diversity, often estimated by the Bray–Curtis index [29].

The first shotgun metagenomic analysis was realized in 2006 [30]. This approach provides information on the overall composition of GIM, with the possibility to predict functions assumed by the diverse bacterial taxa, reliably. Shotgun still requires more complex bioinformatics and computational efforts than the 16S rDNA approach.

Studies on the GIM are often associated with quantitative PCR (qPCR) approaches that are designed to detect and quantify genes of interest. This approach achieves higher specificity and sensitivity compared to 16S rDNA and shotgun sequencing. It is relatively inexpensive and does not require sophisticated bioinformatics skills [31].

Irrespective of the molecular biology approach, important biases in GIM studies can derive from sampling and conservation. Bad storage of stools (temperature, +/– buffer) can induce DNA degradation and thus reduce the quality of downstream analysis. Other important biases could be introduced during DNA extraction from feces. PCR inhibitors in DNA extracted from animals’ stools are particularly difficult to remove. For all these reasons, the International Human Microbiome consortium has provided standards for samples collection, storage, and DNA extraction procedures [32–37]. These standardizations should be adapted also for GIM studies in animals to improve harmonization of methodologies and allow comparison among studies.

Finally, some studies analyzing the effects of antibiotics on GIM have exploited animal models [38] and also bioreactors mimicking the GIM [39]. This approach allows a controlled quantification of antibiotics to which bacteria are exposed but often relies on a simplification of the bacterial community, thus impairing ecological conclusions of antibiotics action on GIM.

Current knowledge on animals GIM and antibiotic interactions is blurred by diversity of posology, administration routes, and implemented methodologies for its analysis. Besides, the pharmacodynamics of each antibiotic varies according to the animal host [40], rendering the picture more complex compared to humans [41].

In this review, we analyzed studies conducted on animals, focusing on the main food-producing and companion animals. The main findings reported on the action of antibiotics on ARGs selection, but also on the GIM composition of animals, were summarized. The analysis encompasses diverse classes of antibiotics, highlighting differences according to the molecule, the administration route, and the methodology applied for the AMR and GIM analysis. The search for original research papers in the PubMed library, accessed during July–August 2021, was conducted using the keywords “antibiotics [animal host] microbiota”. Animal host consisted of calves, bovines, pigs, poultry, ovine, rabbits, horses, dogs, and cats. No studies were found for ovine and rabbits. Seventy-one papers were considered as containing relevant data, as relative studies were conducted analyzing the GIM and ARGs before and after the antibiotic therapy. Studies including the analysis of antibiotic effects on one species or genus, or an ARG, were included, as well.

The summary of the studies highlights: (i) if animals were hosted in experimental or commercial farms for food-producing animals; (ii) the administration route and posology

of the antibiotics; (iii) the methodology applied for analyzing the GIM composition and ARGs; and (iv) main observed effects.

In the following paragraphs, the content is organized according to the animal host and, when pertinent, for each host according to the antibiotic class.

2. Calves

The main components of calves' GIM are *Ruminococcaceae* and *Lachnospiraceae* (40%) (Firmicutes phylum), and *Bacteroidaceae* (15%) (Bacteroides phylum), followed by Enterobacterales (25%) (Proteobacteria phylum), which decreases during GIM maturation (5%), whereas *Prevotellaceae* increases (20%) (Bacteroidetes phylum). The composition of feed-colostrum and GIM in neonate calves is similar, and GIM's evolution occurs rapidly during the first 10 weeks of life [42–44].

Amounts of ARGs were found higher in calves than in adult animals reared in the same environment. Living conditions, such as wet soil and the number of cattle residing in the farm (>500), were risk factors for colonization with cefotaxime (third generation cephalosporin, GC) resistant bacteria [45]. A decrease of Enterobacterales during the first weeks of life has been associated with a general decrease in ARGs abundance in calves, with breed influencing the abundance of certain ARGs and *ampC* gene (copy number) [45].

2.1. Effect of Waste Milk Feeding on Calves' GIM

Exposure of calves GIM to antibiotics is frequent through feeding waste milk containing antibiotics residual. Penati et al. [46] observed that calves fed with milk containing residual cefalexin (first GC) differed for GIM composition, with a final higher abundance of Chlamydiae phylum compared to an untreated group until 6 weeks after cefalexin residual fed withdrawal. Dupouy et al. [47] investigated the selective power of cefquinome (fourth GC) administered to calves colonized by different amounts of Extended-Spectrum-Beta-Lactamase (ESBL) producing *E. coli*. The administration mimicked residual concentration found in waste milk (2 mg/L) and that of udder milk of treated cows (20 mg/L). Cefquinome had a selective effect, irrespective of the administrated concentration, on all calves colonized by ESBL-producing *E. coli* prior to antibiotic exposure. Maynou et al. [48] compared the GIM of Holstein calves receiving raw milk and a formula with waste milk containing residues of beta-lactams and probably lincosamides at unknown concentrations. No difference between the two groups was observed in terms of GIM composition. Effects of low-concentration antibiotics (penicillin, ampicillin, and oxytetracycline) were associated with a decrease of certain microbial functions, such as stress response, regulation of the cell signaling, and nitrogen metabolism, in neonatal GIM of treated calves, potentially affecting the adaptation of GIM to environmental challenges [49]. In another study, a very low concentration of a cocktail of antibiotics in waste milk did not alter calves' GIM composition at the phylum level. Besides, a significant decrease of the *Veillonella* genus was observed in calves exposed to antibiotic residues compared to an unexposed control group [50].

2.2. Therapeutic Concentration of Antibiotics in Calves

2.2.1. Beta-Lactams

In 6-months-old Norwegian Red calves treated by intramuscular injection (IMI) of benzyl-penicillin, Grønbold et al. [51] observed the emergence in *E. coli* of resistance to benzyl-penicillin and to other classes of antibiotics, whereas no resistance was observed in non-treated calves. Considering that *E. coli* has low permeability to benzyl-penicillin, the mechanisms underlining the emergence of such resistance remain unclear.

Antibiotic therapy based on trimethoprim-sulfamethoxazole, a folic acid synthesis inhibitor, followed by ceftiofur (3rd GC), delayed diversification in species composition of calves GIM, whereas inter-individual variability, which usually decreases with maturation, remained overall elevated, suggesting that antibiotics delayed maturation of the GIM [52].

2.2.2. Original Data on the Analysis of Amoxicillin Effects on Calves' GIM

History of amoxicillin therapy has been associated to the rise of resistance in *E. coli* isolates from calves' feces [53]. To our knowledge, longitudinal studies analyzing the effects of amoxicillin on calves' GIM were lacking. We thus prospectively collected feces from calves ($n = 16$) aged from 5 to 26 days, belonging to five breeds (Charolais/Montbeliard, Montbeliard, Prim' Holstein, Charolais/Prim' Holstein, Limousin/Montbeliard), and resident in different farms ($n = 7$) in the Rhône-Alpes region (France), during the period October 2018–March 2020. Eleven out of 16 calves were suffering from omphalitis (umbilical cord infection) and were treated with IMI of amoxicillin (Longamox[®], 15 mg/kg) for a duration varying between 4 and 16 days. The remaining five calves did not receive antibiotic treatment, and their feces were sampled at the same pace of the treated ones. The abundance of 41 ARGs, *intI1*/2/3, and of 16S rDNA was analyzed by qPCR [54]. Seventeen out of 41 investigated ARGs were found in the feces of all calves before amoxicillin treatment. The *bla*_{TEM} gene and bacterial abundance were comparable between the treated and untreated group before treatment (ratio *bla*_{TEM}/16S rDNA: 0.013 in both groups). At the end of amoxicillin treatment (T1), the amount of *bla*_{TEM} increased in treated calves (*bla*_{TEM}/16S rDNA ratio: 0.040) along with other ARGs (*tetA*, *strA* and *strB*), and *intI1*, index of class 1 integrons. These data suggest co-selection by amoxicillin treatment of ARGs related to other antibiotic classes and potential multidrug development (Figure 1). A decrease of all ARGs was observed 1 week after amoxicillin withdrawal (T2) (*bla*_{TEM}/16S rDNA ratio: 0.008). The amount of *bla*_{TEM} constantly decreased in the untreated group (*bla*_{TEM}/16S rDNA ratio: T1, 0.005; T2: 0.002). The difference observed in the amount of ARGs at pretreatment and post-treatment, or between treated and untreated group, was not statistically significant (Wilcoxon Mann Whitney or Wilcoxon signed-rank test, $p > 0.05$). Several factors could confound the effect of the amoxicillin treatment on the *bla*_{TEM} amount, for instance variation of the *bla*_{TEM} gene amount among individuals, probably due to variation of calves' age, which ranged from 5 to 26 days. Age is a determinant for GIM composition and ARGs amount at early life. In addition, calves were distributed in seven commercial farms probably contributing to the difference in ARGs content as well, because of different farm management. Environmental exposure of all calves to ARGs cannot be excluded, as calves of the untreated and treated group lived in the same farm, thus probably influencing the level of difference of *bla*_{TEM} amount between the two groups. For a better understanding of antibiotics action on the GIM and selection of ARGs, experiments in environmentally controlled set-up would be a benefit for avoiding confounding factors influencing GIM composition and ARGs variation further than antibiotic action. However, studies in commercial farms are necessary to model antibiotic therapies effects in a real-life environment and evaluate ARGs propagation to other hosts or in the farm environment.

2.2.3. Macrolides

Prophylactic subcutaneous injection (SCI) of tulathromycin caused a decrease of *Bifidobacterium* genus (Actinobacteria) in treated calves [55]. On the contrary, comparison of metaphylactic therapy based on enrofloxacin, a fluoroquinolone, or tulathromycin in Holstein calves did not evidence major changes in the GIM neither at the phylum level nor for gene function. Besides, Desulfocivibionales (Proteobacteria), which include species of potential pathogens for humans [56], had a higher relative abundance in the enrofloxacin-treated group 56 days post-withdrawal [57].

Metaphylactic SCI of tildipirosin did not alter the GIM of Holstein calves, at least at the phylum level [58]. Several antibiotic therapies caused a decrease of GIM diversity and *E. coli* amount during the treatment and until 15 days after withdrawal in Holstein calves observed in three different commercial farms [59].

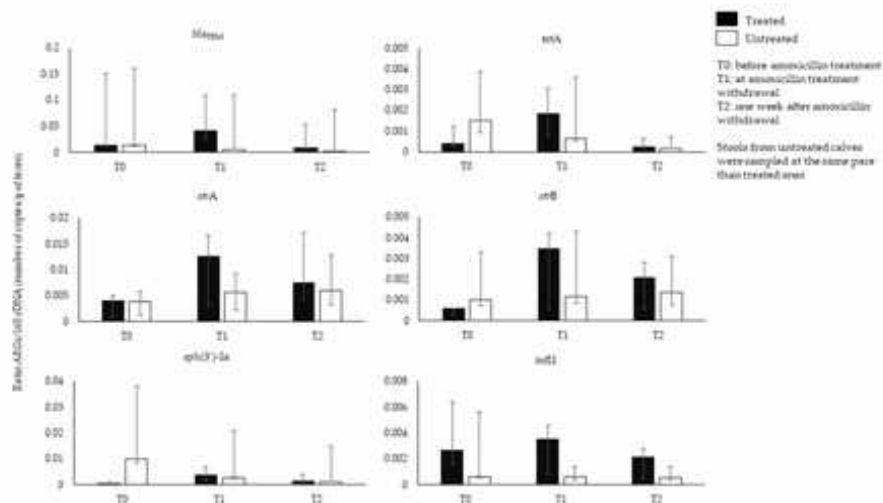


Figure 1. Representation of ARGs/16S rDNA ratio (number of copies/g of feces) evaluated by qPCR in feces of treated and untreated calves at amoxicillin pre-treatment (T0), at amoxicillin withdrawal (T1), and 1 week after amoxicillin withdrawal (T2).

2.2.4. Tetracyclines

Keijser et al. [60] analyzed the effects of a low and high dose of oxytetracycline in treated calves. The high-dose was administered for 5 days, whereas the low-dose was administered for 7 weeks. Major changes compared to a group of untreated animals were observed over time. Both oxytetracycline doses correlated with a decrease of *Ruminococcus*, *Coprobacillus*, and *Lachnospiraceae*, all belonging to the Firmicutes phylum, along with an increase of *Prevotella* (phylum Bacteroidetes), *Faecalibacterium*, and *Blautia* (phylum Firmicutes), compared to an untreated group. The selection of *tetM* gene and other ARGs, such as *mel* and *floR*, occurred only in high-dose treated calves and lasted for all the study period (42 days). Oultram et al. [61] analyzed the effects of oxytetracycline (IMI), tulathromycin (SCI), and florfenicol (SCI) used to treat pneumonia and otitis occurring in 7-week-old Holstein calves, hosted in a commercial farm. Considering the five most abundant detected genera, *Lactobacillus*, *Faecalibacterium*, *Bacteroides*, *Parabacteroides*, and *Sharpea*, a statistically significant decrease in the oxytetracycline-treated calves was observed for *Lactobacillus* genus compared to the control group. Overall, antibiotic treatment slightly decreased species richness in the calves' microbiota 1 week after withdrawal. However, no statistical significance was observed compared with control group. Thames et al. [13] studied by qPCR the effects of neomycin and tetracycline orally administered on the abundance of selected ARGs (*tetC/G/O/W/X*, *ermB/E*, *sul1/2*; *int11*), and found that only *tetO* was significantly more abundant in the treated group.

2.2.5. Other Antibiotics

Lhermie et al. [62] analyzed the effect of fluoroquinolones at low (2 mg/L) and high (10 mg/L) doses administered by IMI in young bulls (7–10-months-old) and calves (2–5-weeks-old). The therapy moderately selected for resistant Enterobacterales compared to the untreated group, and with less detectable effects in young bulls, probably because of a more mature GIM compared to calves that was expected to contain less Enterobacterales and more species difficult to cultivate (the study was conducted by cultivation). However, calves were colonized by fluoroquinolone-resistant bacteria before the treatment. Dobrzanska et al. [6] analyzed the effect of thiamphenicol. At 7 days from thiamphenicol administration, Proteobacteria increased because of *E. coli* expansion, along with the emergence of *mcr-2*, a less prevalent gene than *mcr-1* responsible for colistin resistance, and *oqx8*

gene, encoding for antibiotic efflux pump. In the treated group, a rise of methanogenic Archaea and *Prevotellaceae*, typically associated to weight gain, was also observed.

3. Adult Bovines

Studies conducted on adult bovines are scarce. Recently, Wang et al. [63] provided information on the GIM composition and ARGs in yak, beef, and dairy cattle. Composition of the GIM was similar among animals. However, abundance of ARGs differed among hosts, with higher abundance in beef and dairy cattle than in yak.

Holman et al. [64] analyzed the effects of a single IMI dose of oxytetracycline and tulathromycin in Angus-Herford cattle in an experimental farm and moved to a feedlot for the study. The moving to the feedlot caused more remarkable changes in the GIM composition than antibiotic therapies. Besides, both antibiotics caused the decrease of several species compared to the control group, and recovery was observed 12 days post-treatment. The *tetM* and *tetW* genes augmented in treated animals and remained higher than in the untreated control group up to 34 days post-treatment.

In general, studies conducted in calves were more numerous than those conducted on adult bovines, probably because antibiotic therapies are more frequent in young animals that suffer more often than adults from diseases such as pneumonia and diarrhea [45,65] (Table 1).

Table 1. Summary of studies investigating impact of antibiotics on bovines' intestinal microbiota and relative antibiotic resistance genes.

Antibiotic	Farm	Administration	Dose (mg/kg of Animal)	Duration (Days)	Method	Effect on GIM	Effect on ARGs	Reference
Thiamphenicol	Exp	SCI	40	1	16S rDNA (V3–V4), shotgun, qPCR	Proteobacteria, Archaea <i>Prevotellaceae</i> ↑	<i>mcr-2</i> , <i>ngxB</i> ↑	[6]
Neomycin Oxytetracycline	Exp	O	10 mg/day 1000 mg/day	50 14	qPCR	ND	<i>tetO</i> ↑	[13]
Cefalexin	Comm	O	ND	14	16S rDNA (V3–V4)	Chlamydiae ↑	ND	[46]
Cefquinome	Exp	O	2 mg/L 20 mg/L	3	Cultivation	ESBL-producing <i>E. coli</i> ↑	<i>bla_{CTX-M-1}</i> ↑	[47]
Beta-lactams	Exp	O	ND	42	16S rDNA (V1–V3)	None	ND	[48]
Ceftiofur Penicillin Ampicillin Oxytetracycline			0.1 mg/L 0.005 mg/L 0.01 mg/L 0.3 mg/L					
Benzylpenicillin	Exp	IMI	40,000 IU	6–14	Cultivation, ARISA and TRFLP	None	PEN resistance ↑	[51]
Trimethoprim-sulfamethazole	Exp	O	0.7 mL/10 kg	3	16S rDNA (V1–V3)	Diversity ↓ Intra-individual variability ↑	ND	[52]
Amoxicillin	Comm	IMI	15	5–26	qPCR	Abundance ↓	<i>bla_{TEM}</i> , <i>strA/B</i> , <i>tetA</i> , <i>intI1</i> ↑	Personal data
Ceftiofur	Exp	SCI	0.2 mL/10 kg	1	qPCR		ND	[55]
Tulathromycin			2.5			<i>Bifidobacterium</i> ↓		
Tulathromycin Enrofloxacin	Comm	SCI	7.5 and 12.5 2.5	1	Shotgun	Desulfovibrionales ↑	<i>ermA</i> ↑ <i>gyrA</i> mutation ↑	[57]
Tildipirosin	Exp	SCI	4	1	16S rDNA (V4), qPCR	None	ND	[58]
Multiple	Comm	O	ND	1–10	16S rDNA (V4), qPCR	Diversity, <i>E. coli</i> ↓	ND	[59]
			2 g/day	5			<i>tetM</i> , <i>mel</i> and <i>flor</i> ↑	[60]
Oxytetracycline	Exp	O	0.1–0.2 mg/day	42	16S rDNA (V4) and shotgun	<i>Ruminococcus</i> , <i>Coprobacillus</i> , <i>Lachnospiraceae</i> ↓ <i>Prevotella</i> , <i>Faecalibacterium</i> , <i>Blautia</i> ↑	None	

Table 1. Cont.

Antibiotic	Farm	Administration	Dose (mg/kg of Animal)	Duration (Days)	Method	Effect on GIM	Effect on ARGs	Reference
Oxytetracycline Tulathromycin Florfenicol	Exp	IMI SCI	20 40	ND	16S rDNA (V1–V2)	<i>Lactobacillus</i> ↓ None	ND	[61]
Marbofloxacin	Comm	IMI	2 and 10	1	Cultivation	None	FQ resistant Enterobacterales ↑	[62]
Oxytetracycline Tulathromycin	Exp	IMI	20 2.5	1 5–14	16S rDNA (V4), qPCR	Diversity, abundance ↓ <i>Dialister</i> , <i>Oscillospira</i> , <i>Roseburia</i> , <i>Lachnospiraceae</i> ↓	<i>tetM</i> , <i>tetW</i> ↑	[64]*

Note. GIM: gastro-intestinal microbiota; ARGs: antibiotic resistance genes; Exp: experimental; O: oral; ND: not determined; IMI: intramuscular injection; PEN: penicillin; Comm: commercial; ESBL: extended spectrum beta-lactamase; SCI: sub-cutaneous injection; FQ: fluoroquinolones. * this study was conducted on adult animals, whereas all the others were conducted on calves. †: increase; ↓: decrease.

4. Pigs

A reference catalogue of pigs' GIM is available, and the basal resistome of pigs never exposed to antibiotics and residing in experimental farms was provided [66–68]. Tetracycline resistance genes are the most abundant in pigs' resistome, whereas the main components of the microbiota, at the phyla level, are Firmicutes (65.5%), Bacteroidetes (14%), Proteobacteria (10%), and Actinobacteria (7.1%). Piglets have been used as a model for studying neonatal entero-colitic diarrhea [69], probably justifying the large amount of available studies analyzing the GIM.

4.1. Beta-Lactams

Effects of antibiotics on pigs' GIM have been investigated since the early 2000s, exploiting DGGE (Denaturing Gradient Gel Electrophoresis). Besides the limitations inherent to this technique [70], studies converged for effects of IMI-administered amoxicillin, indicating a decrease of Firmicutes and of species diversity and abundance, along with an increase of Proteobacteria in GIM of treated piglets [71,72]. Fohse et al. [73] investigated the therapeutic effects of amoxicillin administration in piglets with a specific focus on immune system development. A transient increase of Proteobacteria and a decrease of Firmicutes, along with decreased alpha-diversity, were observed in treated animals compared to the untreated group. These differences disappeared 3 days after the withdrawal of the therapy. However, in this study, the administration route of amoxicillin and the region of 16S rDNA sequenced were not explicit. Massacci et al. [74] investigated the effect of amoxicillin in weaned piglets suffering from *E. coli* intestinal infection and compared the composition of the microbiota between piglets treated either orally or by parenteral injection, and an untreated control group. Oral administration of amoxicillin produced a dramatic decrease of *Lactobacillus* spp., *Prevotella copri*, and *Ruminococcus*, which are crucial genera for the fiber metabolism, compared to parenteral administration and control groups. After amoxicillin withdrawal, *Lactobacillus* spp. remained more abundant in the control group compared to the two treated groups. Bibbal et al. [75] demonstrated by qPCR that *bla*_{TEM}, responsible for ampicillin resistance, increased in feces of pigs after ampicillin administration compared to an untreated group. Furthermore, oral administration significantly increased *bla*_{TEM} excretion compared to the IMI administration. Connelly et al. [76] compared by shotgun sequencing the effect of oral administration of amoxicillin versus intra-venous injection (IVI) of ertapenem. The two antibiotics altered the GIM in different ways: both antibiotics reduced the relative amount of *Faecalibacterium*, a main butyrate-producing genus, *Megasphaera*, *Oxalobacter*, a genus contributing to good health status in humans, but amoxicillin also affected *Lactobacillus* spp.; amoxicillin increased the amount of *Escherichia*, *Bacteroides*, *Fusobacterium*, *Shigella*, and *Klebsiella* genera, suggesting the emergence of potential gastro-intestinal pathogens. Ertapenem increased the

relative abundance of *Bacteroides*, *Pseudomonas*, *Enterococcus*, and *Acinetobacter* genera, to which potential gastro-intestinal and extra-intestinal pathogens belong to, as well. Both antibiotics selected ARGs. Surprisingly, the results were not compared to those of a non-treated group of piglets. Using a control, untreated groups should not be neglected in the experimental set-up especially when analyzing GIM and ARGs of young animals in which GIM is not mature and evolve rapidly. Kouadio et al. [77] studied the effect of amoxicillin administered to piglets orally and by IMI (dose not indicated in the study). By cultivation on selective and non-selective media, the ratio between amoxicillin-resistant and susceptible Enterobacterales was not statistically different when comparing the two administration routes but significantly different between treated and non-treated animals, highlighting the selection of resistant bacteria by amoxicillin whatever the administration route. Lin et al. [78] demonstrated that sub-therapeutic doses of ceftiofur and enrofloxacin caused a higher selection than therapeutic doses of *E. coli* resistant to these antibiotics in feces of challenged piglets, irrespective of administration route (IMI or oral). Yun et al. [79] did not find selection of amoxicillin-resistant *E. coli* in piglets treated by amoxicillin (IMI). However, in the latter study, pigs were hosted in commercial farms where the amoxicillin effect could have been confounded by other variables.

4.2. Macrolides

IMI of tulathromycin in neonatal piglets did not induce significant differences in diversity of GIM or ARGs abundance compared to the untreated control group [80]. On the contrary, a decrease in the diversity of pigs' GIM composition was observed after lincomycin in-feed administration, favoring the relative raise of Firmicutes and Actinobacteria and decrease of Bacteroidetes and Spirochetes. In particular, a decrease of genera involved in fibers metabolism (*Triponema*, *Succinivibrio*, *Fibrobacter*, and *Cellulosilyticum*) was observed, in favor of an increase of potentially pathogenic genera (*Clostridium*, *Aerococcus*, *Escherichia*, and *Corynebacterium*) in lincomycin-treated pigs [81].

4.3. Tetracyclines

Oral administration of oxytetracycline induced an increase of Bacteroidetes and Proteobacteria by expansion of *Prevotella* and *Escherichia* genera, whereas a decrease of Firmicutes occurred along with diversity and species richness. ARGs were found increased during treatment, with a decrease sometimes observed after treatment withdrawal [82]. In-feed chlortetracycline in weaned piglets induced a lower species diversity of GIM composition, with an increase of *Lactobacillus* and *Pseudoalteromonas* along with a decrease of *Prevotella*, *Sphaerochaeta*, and *Shuttleworthia* genera. An increase in the abundance of the tetracyclines resistance genes *tetC/G/Q/W*, along with *sul1/2* and *int1/2*, was observed in chlortetracycline feed piglets compared to untreated controls [83]. Therapeutic effects of oxytetracycline were studied on piglets to compare IMI and oral administration [16]. In this study, Ricker et al. observed a decrease of Fibrobacteres and Proteobacteria together with an increase of Euryarchaeota and Actinobacteria in GIM of orally-treated piglets, while mild effects were observed in IM-injected piglets. Furthermore, in the oral-administered group, enrichment of genes conferring tetracycline resistance (*tetW*) was observed, and of *aph2'*-id aminoglycosides resistance gene, suggesting a co-selection process. Holman et al. [84] reported a decrease of diversity and richness of the piglets GIM receiving chlortetracycline orally. Zhang et al. [85], comparing the effects of chlortetracycline with those of *Lactobacillus* administration and a non-treated group, showed that Verrucomicrobia that are involved in human gut health homeostasis were less abundant in the chlortetracycline-treated group.

4.4. Other Antibiotics

Using IMI as a unique administration route, Zeineldin et al. [86] analyzed the effects of a single dose of different antibiotics, including penicillin, ceftiofur (free acid or conjugated with hydrochloride acid), oxytetracycline, and tulathromycin. All antibiotics caused a shift in diversity and richness of the GIM with tulathromycin and ceftiofur free acid, causing a

significant decrease of the relative abundance of the Bacteroidetes phylum. The resilience to a pre-treatment composition was not completely achieved after 14 days for any of the treatments, but especially for ceftiofur free acid and oxytetracycline. The study did not include a non-treated control group.

Oral treatment with colistin of piglets induced a decrease of potential entero-pathogens such as *E. coli* or *Shigella* spp., limited to the study period. No mention on AMR effect of colistin administration was reported [87]. Fleury et al. [88] compared the effect of oral administration of colistin in weaned piglets at low and high doses. The major evidence consisted in a decrease of the *E. coli* population in the group treated with a high dose, an effect that disappeared at withdrawal. No selection of resistance was observed. This is of relevance for the global therapeutic arsenal, considering that colistin is used as a last-line resort antibiotic in humans and the emergence of plasmid-located resistance mechanisms [89] has put in discussion the usage of this drug in animals. However, long-course colistin therapies could produce different results on ARGs selection.

Pisetti et al. reported an increase of Firmicutes together with a decrease of Bacteroidetes, from piglets treated with high antibiotic dosage in cocktail [90]. In this study, piglets not receiving antibiotics were hosted in a separated experimental farm, compared to study groups, which were hosted in commercial farms. Development of multidrug resistance in cultivable *E. coli* and enterococci positively correlated with the amount of antibiotic administered to piglets. In-feed flavomycin combined with enramycin was associated to a lower relative abundance of Proteobacteria and Fibrobacteres compared to antibiotic-free pigs [91]. In-feed tylosin was associated to an increase of the Firmicutes/Bacteroidetes ratio and a decrease of Tenericutes compared to control piglets [92] (Table 2).

Table 2. Summary of studies investigating impact of antibiotics on pigs' intestinal microbiota and relative antibiotic resistance genes.

Antibiotic	Farm	Administration	Dose (mg/kg of Animal)	Duration (Days)	Method	Effect on IM	Effect on ARGs	Reference
Oxytetracycline	Exp	O	5	7	16S rDNA (V4), and qPCR	Fibrobacteres, Proteobacteria ↓ Euryarchaeota, Actinobacteria ↑	<i>tetW</i> , <i>aph2'-id</i> ↑	[16]
		IMI	4					
Amoxicillin	Exp	IMI	15	1	DGGE of 16S rDNA	Diversity, abundance ↓	ND	[71]
Tilmicosin	Exp	O	400	ND	DGGE of 16S rDNA	Abundance, Enterobacteriales ↓ Lactobacilli ↑	ND	[72]
Amoxicillin Doxycycline			600 300					
Amoxicillin	Exp	O	30	14	Cultivation, 16S rDNA	Diversity, Firmicutes ↓ Proteobacteria ↑	ND	[73]
Amoxicillin	Exp	PAR	15	5	Cultivation, 16S rDNA (V3–V4)	<i>Lactobacillus</i> ↓	ND	[74]
Ampicillin	Exp	O	12–20					
		IMI	20	7	qPCR	ND	<i>bla</i> _{T1M} ↑	[75]
Amoxicillin	Exp	PAR	20	7	Shotgun	<i>Lactobacillus</i> , <i>Faecalibacterium</i> , <i>Megasphaera</i> , <i>Oxalobacter</i> ↓ Enterobacteriales, Bacteroides, <i>Fusobacterium</i> ↑ <i>Faecalibacterium</i> , <i>Megasphaera</i> , <i>Oxalobacter</i> ↓	<i>cfxA</i> , <i>bla</i> _{T1M} , <i>aph4-1a</i> , <i>sat-2a</i> , <i>sph</i> , <i>strA/B</i> , <i>mplE</i> , <i>sul2</i> , <i>tetB</i> , <i>tetY</i> ↑	[76]
Ertapenem		IVI	50			<i>Bacteroidetes</i> , <i>Pseudomonas</i> , Enterococcus, <i>Acinetobacter</i> ↑	<i>bla</i> _{IMP27} , <i>aph4-1a</i> , <i>SAT-2a</i> , <i>sph</i> , <i>strA/B</i> , <i>mplE</i> , <i>sul2</i> , <i>tetB</i> , <i>tetY</i> ↑ <i>dfrA5/12</i> ↓	

Table 2. Cont.

Antibiotic	Farm	Administration	Dose (mg/kg of Animal)	Duration (Days)	Method	Effect on IM	Effect on ARGs	Reference
Amoxicillin	Exp	IMI or O	ND	5	Cultivation	ND	AMX-resistant <i>E. coli</i> ↑	[77]
Ceftiofur	Exp	PAR	0.5–5	3	Cultivation	ND	Resistant <i>E. coli</i> ↑	[78]
Enrofloxacin		O	1–10	3				
Amoxicillin	Com	IMI	150 mg/ml	1	Cultivation	ND	None	[79]
Tulathromycin	Exp	IMI	2.5	1	Shotgun sequencing	None	None	[80]
Lincomycin	Com	O	1.000	7–14	16S rDNA (V3–V4)	Diversity, abundance, Spirochetes, Bacteroidetes ↓, Firmicutes, Actinobacteria ↑	ND	[81]
Oxytetracycline	Exp	O	40	14	Metagenomic shotgun sequencing	Diversity, richness, Firmicutes ↓, Bacteroidetes, Proteobacteria ↑	Enrichment, diversity ↑	[82]
Chlortetracycline	Exp	O	75	90	16S rDNA (V3–V4), and qPCR	<i>Pseudoalteromonas</i> ↑, <i>Prevotella</i> , <i>Sphaerochaeta</i> , <i>Shuttleworthia</i> ↓	<i>tetC</i> , <i>tetG</i> , <i>tetW</i> and <i>sulI</i> ↑	[83]
Chlortetracycline	Exp	O	400	12	16S rDNA (V4)	Diversity, richness, <i>Lactobacillus</i> , <i>Succinivibrio</i> ↓	ND	[84]
Chlortetracycline	Exp	O	100	10	16S rDNA (V3–V4)	Verrucomicrobia ↓	ND	[85]
Ceftiofur (FA)	Exp	IMI	5	ND	16S rDNA (V1–V3)	Firmicutes/Bacteroidetes ratio: ↑	ND	[86]
Ceftiofur (Na)			5					
Oxytetracycline			4					
Penicillin			15.000 UI/lb					
Tulathromycin	Exp	O	2.5	5	16S rDNA (V4)	↑	ND	[87]
Colistin			50.000 UI/kg					
Cocktail	Exp/Comm	O	3.600 UI/kg	5	16S rDNA (V4), qPCR	Enterobacteriales, Enterococcaceae ↑	ND	[88]
Cocktail	Com	ND	Multiple doses	1–66	16S rDNA (V1–V3), cultivation	Firmicutes ↑, Bacteroidetes ↓	<i>E. coli</i> and <i>Enterococcus</i> MDR ↑	[90]
Flavomycin	Comm	O	5	56	16S rDNA (V3–V4)	Proteobacteria, Fibrobacteres ↓	ND	[91]
Enramycin			15					
Tylosin	Exp	O	100	39	16S rDNA (V3–V4)	Firmicutes/Bacteroidetes ↑, Tenericutes ↓	ND	[92]

Note. GIM: gastro-intestinal microbiota; ARGs: antibiotic resistance genes; Exp: experimental; O: oral; ND: not determined; IMI: intramuscular injection; DGGE: denaturing gradient gel electrophoresis; PAR: parenteral; Comm: commercial; IVI: intravenous injection; MDR: multidrug resistant; ↑: increase; ↓: decrease.

5. Poultry

The predominant phyla occurring in poultry GIM are Firmicutes and Bacteroidetes, followed by Proteobacteria and Actinobacteria, with a proportion of Firmicutes and Bacteroidetes increasing relatively to Proteobacteria with age [93,94]. The easier accessibility to the gastro-intestinal organs by dissection has permitted to better define species components of the different compartments compared to other animals. Diversity of GIM composition increases from the crop to the colon, at least in adult hens [95]. Relevant differences in the GIM composition can be related to breeding management. Indeed, in free-range chicken, Bacteroidetes are the most abundant taxa, whereas conventional-range chicken Firmicutes, and ARGs typically found in Firmicutes, are relatively more abundant [94]. In poultry, more antibiotic classes have been analyzed compared to bovines and pigs, including fluoroquinolones.

5.1. Beta-Lactams

Penicillin administered as feed additive to 1-day-old chickens resulted in a higher relative abundance of Firmicutes compared to Bacteroidetes at 18 days. Penicillin-fed chicks had higher body weight compared to chickens not receiving the antibiotic [96]. Ampicillin interfered differently with GIM for composition and ARGs selection according to administration routes in Leghorn chickens. Oral administration had a higher impact on GIM modification, causing augmentation of Proteobacteria at the expense of Firmicutes phylum. This shift was attributable to an increase of Enterobacterales and in this order by *Klebsiella* and *Escherichia* genera. Higher increase of ARGs was observed with oral treatment compared to IMI, although no statistical results were reported for the two routes and relatively to the control group [97].

5.2. Streptogramins

Chen et al. [98] compared the effect of in-feed virginiamycin and plant extracts oil on the caecum of young Cobb chickens. Chickens fed with virginiamycin presented lower species diversity compared to oil-fed and control groups, with an increased relative abundance of the Bacteroidetes phylum along with a decrease of Firmicutes. Changes in the metabolome of the caecum were noticed compared to the control group. Dumonceaux et al. [99] previously analyzed the effect of in-feed virginiamycin in the caecum of 50-days-old Cobb chickens. The effect on the diversity of species was less relevant than in the distal part of the intestine compared to the proximal one. In combination with monensin, an anticoccidial, virginiamycin caused a significant increase of the *Escherichia* and a decrease of the *Roseburia* genera relatively to the untreated control in Ross chickens [100].

5.3. Tetracyclines

In Lohmann Brown hens hosted in an experimental farm, oral treatment by single or repeated doses of tetracycline and streptomycin caused a decrease in GIM species diversity at 48 h hours post-treatment for both regimens. The sequences representative of enterococci and *E. coli* raised, suggesting potential dysbiosis. Restoration of GIM composition to pre-treatment was observed soon after withdrawal. Samples from tetracycline-treated and streptomycin-treated hens were pooled for sequencing analysis, an advantageous strategy for optimizing experimental costs and probably simplifying bioinformatics analysis. However, results obtained for tetracycline or streptomycin-treated chickens were not compared to an untreated control group [93].

In-feed chlortetracycline on the GIM of 42-days-old Arbor Acre chickens caused an augmentation of species diversity in the Firmicutes and Actinobacteria phyla, whereas that of Proteobacteria decreased. An increase of the *Lactobacillus* genus, typically considered as benefic to host health, was observed in the chlortetracycline-treated group [101]. The effect of chlortetracycline, in combination with virginiamycin and amoxicillin, was investigated by Banerjee et al. [102], using sub-therapeutical doses of antibiotics. Compared to a control group fed without antibiotics, Firmicutes, and notably lactobacilli, were relatively more abundant than Bacteroidetes in the antibiotic-fed group. This composition correlated with an increased weight gain in antibiotic-fed chickens.

5.4. Fluoroquinolones

Effects of different doses of enrofloxacin on GIM and resistance to *Salmonella* colonization were investigated by Ma et al. [103]. Enrofloxacin-treated chickens demonstrated higher colonization and invasion by *S. Typhimurium*. Furthermore, chickens treated with a high dose had lower abundance of genera beneficial to host health including *Anaerotruncus*, *Butyrivibrio*, and *Ruminococcus* compared to the untreated group. Li et al. [104] analyzed the effects of repeated cycles of enrofloxacin administrations on *S. Typhimurium* challenged chickens. High enrofloxacin dosage eradicated *S. Typhimurium* shedding and caused significant GIM changes compared to low-dosage treatments and untreated groups, with a major increase of *Lactococcus*, *Bacillus* and of Proteobacteria (*Burkholderia*,

Pseudomonas, *Rhizobium*, and *Acinetobacter* genera). Enrofloxacin at high and low dosage caused the decrease of *Anaerotruncus* genus along with *Blautia*, *Janibacter*, *Flavisolibacter*, and *Parasuterella*, which did not return to the baseline at 7 days post withdrawal. The effects of enrofloxacin on protection against *S. Typhimurium* colonization and invasion were contrasting according to the studies from Ma and Li [103,104]. However, methods to recover *S. Typhimurium* in challenged hosts were different, as well as antibiotic regimen (Li et al. administered repeated antibiotic doses). However, the two studies converged in reporting dramatic changes in the GIM composition of treated animals. Another study found the effect of enrofloxacin consisting in a decrease of microbiota richness, to be transient when administered to 2-week-old Ross chickens, similarly to amoxicillin [105]. In 1-month-old Jing Hong GIM chickens, Elokil et al. [106] studied the effects of enrofloxacin combined with diclazuril, an anticoccidial drug. Overall, Firmicutes, Actinobacteria, Thermi, and Verucomicrobia phyla decreased in treated chickens, and return to baseline was not observed even at 15 days after withdrawal of treatment.

5.5. Bacitracin

Johnson et al. [95] compared the effect of bacitracin administered at sub-therapeutic and therapeutic doses to turkeys. A decrease of species diversity in the caecum was observed with both dosages, although a more dramatic decrease was obtained with therapeutic dose. This difference disappeared over time (42 days). Alteration of the GIM composition was associated to metabolome alteration in treated turkeys, inducing changes potentially beneficial to turkeys' health. Previously, Díaz Carrasco et al. [107] compared the effects of sub-therapeutic bacitracin to tannins in the caecum of Cobb chickens, sampled at different ages (12, 26, and 30 days old). Differences in species richness were age-dependent. However, in bacitracin-fed chickens, lower species richness was observed at 30 days compared to control and tannins-fed groups, where Firmicutes abundance relatively to Bacteroidetes was higher. Proctor and Phillips [108] analyzed the effects of bacitracin at therapeutic dosage in the colon and caecum microbiota of 30-days-old Cornish/Rock chickens. Both treated and untreated control groups demonstrated a GIM composition mainly constituted of Firmicutes, followed by Proteobacteria, Actinobacteria, and Bacteroidetes. This latter phylum, differently from other studies, occurred with a relative abundance < 1%. Effects of bacitracin concerned mostly the colon microbiota. In the caecum, at the class level, Clostridia increased, whereas *Peptostreptococcaceae* decreased.

5.6. Other Antibiotics

Avilamycin caused decreased diversity in the ileum of treated chickens compared to the control group, while no difference to control was observed at the caecum level [109].

Prophylactic administration of several antibiotics to Ross chickens resulted in modification of the ileum and caecum GIM. In particular, an increase of *Enterococcaceae* was observed in chickens treated with amoxicillin or thiamphenicol [110] (Table 3).

Table 3. Summary of studies investigating impact of antibiotics on chickens' intestinal microbiota and relative antibiotic resistance genes.

Antibiotic	Farm	Administration	Dose (mg/kg of Animal)	Duration (Days)	Method	Effect on GIM	Effect on ARGs	Reference
Tetracycline	Exp	O	60	7	16S rDNA (V3–V4), qPCR	Bifidobacteriales, Bacteroidales, Clostridiales,	ND	[93]
Streptomycin			15	2		Desulfovibrionales, Burkholderiales, Campylobacteriales ↓, Enterobacteriales, Lactobacillales ↑		
Bacitracin	Exp	O	50 200	35 77	16S rDNA (V1–V3)	Diversity ↓ (caecum)	ND	[95] *
Penicillin	Exp	O	55	18	Pyrosequencing, qPCR	Firmicutes ↑ Bacteroidetes ↓	ND	[96]

Table 3. Cont.

Antibiotic	Farm	Administration	Dose (mg/kg of Animal)	Duration (Days)	Method	Effect on GIM	Effect on ARGs	Reference
Ampicillin	Exp	IMI or O	300	5	qPCR, 16S rDNA, (V4–V5), shotgun	Proteobacteria ↑	None	[97]
Virginiamycin	Exp	O	30	28	16S rDNA (V3–V4)	Diversity, richness, Firmicutes ↓ Bacteroidetes ↑	ND	[98]
Virginiamycin	Exp	O	20	50	Cultivation	<i>Lactobacillus</i> , <i>Clostridiotes</i> , <i>Globicatella</i> , <i>Enterococcus</i> , <i>Corynebacterium</i> ↑	ND	[99]
Monesin Virginiamycin Tylosin	Comm	O	110 110 15–20	14	16S rDNA (V3), shotgun	Firmicutes ↓ Proteobacteria ↑	None	[100]
Chlortetracycline	Com	O	100	42	16S rDNA (V1–V9)	<i>Lactobacillus</i> , <i>Megamonas</i> , <i>Helicobacter</i> ↑ <i>Alistipes</i> ↓	ND	[101]
Amoxicillin Chlortetracycline Virginiamycin	Exp	O	0.50 µg/kg 0.1 0.015	42	Cultivation, 16S rDNA (V1–V2)	Firmicutes/ Bacteroidetes ↑	ND	[102]
Enrofloxacin	Exp	O	10 or 100	7	16S rDNA (V3–V4)	<i>Anaerotruncus</i> , <i>Butyrivococcus</i> , <i>Ruminococcus</i> ↓	ND	[103]
Enrofloxacin	Exp	O	10 100	7	16S rDNA (V4)	<i>Anaerotruncus</i> , <i>Blautia</i> , <i>Janibacter</i> , Flavisolibacter, Parasutterella ↓ Proteobacteria, <i>Bacillus</i> , <i>Lactococcus</i> ↑ <i>Anaerotruncus</i> , <i>Blautia</i> , <i>Janibacter</i> , Flavisolibacter, Parasutterella ↓	ND	[104]
Amoxicillin Enrofloxacin	Exp	O	5 11	5	16S rDNA (V1–V9)	Diversity ↓	ND	[105]
Enrofloxacin /Diclazuril	Exp	O	10/0.3	14	16S rDNA (V4)	Firmicutes, Actinobacteria, Thermi, Verrucomicrobia ↓	ND	[106]
Bacitracin	Exp	O	1000	30	16S rDNA (V3–V4)	Richness, Firmicutes ↓ Bacteroidetes ↑	ND	[107]
Bacitracin	Exp	O	200	7	16S rDNA (V3–V5)	Caecum: <i>Clostridia</i> ↑, <i>Peptostreptococaceae</i> ↓ Distal colon: <i>Oscillospira</i> , <i>Erysipelotrichaceae</i> ↓, <i>Lachnospiraceae</i> ↑	ND	[108]
Avilamycin	Exp	O	25	35	16S rDNA (V1–V3)	Diversity ↓ (ileum)	ND	[109]
Amoxicillin Thiamphenicol	Exp	O	1.430 0.2	22	16S rDNA (V3–V4)	<i>Enterococaceae</i> ↑	ND	[110]

Note. GIM: gastro-intestinal microbiota; ARGs: antibiotic resistance genes; Exp: experimental; O: oral; Nd: not determined; IMI: intra-muscular injection; *: this study was conducted on turkeys; Comm: commercial; ↑: increase; ↓: decrease.

6. Horses

Horses are considered both food-producing and companion animals, thus they are potential reservoir of AMR for humans by direct contact. In Australia, high occurrence of tetracycline resistance genes was reported [111], whereas in Europe prevalence of third and fourth GC resistance is high in *E. coli* from healthy horses' feces, with medication recognized as risk factors for its occurrence [112]. De Lagarde et al. evidenced also that residing in a riding school and being in contact with >5 caring persons were potential risk factors for ESBL-producing *E. coli* colonization of horses.

Benzyl-penicillin effect by IMI was evaluated on hospitalized horses. Development of resistance in *E. coli* could not be firmly attributed to antibiotic therapy, considering that resistance also developed in *E. coli* from non-treated horses. Differences in GIM composition after benzyl-penicillin treatment were not reported, while an increase of Bacteroidetes,

Clostridioides perfringens, and enterococci was observed after hospitalization [113]. Costa et al. [7] investigated the effects of penicillin, ceftiofur, and trimethoprim-sulfadiazine on the GIM of mares. Trimethoprim-sulfadiazine caused the most relevant reduction of Verrucomicrobia amount. However, since trimethoprim-sulfadiazine was the only drug administered orally, a real comparison among the different molecules was hindered. At treatment withdrawal (25 days), Proteobacteria amount decreased along with an increase of Firmicutes, suggesting resilience of the GIM. Harlow et al. [114] enumerated colonies of cellulolytic bacteria, lactobacilli, *Salmonella*, and *Clostridioides difficile*, when GIM of horses was challenged by IVI of ceftiofur or oral trimethoprim-sulfadiazine. Both antibiotic administrations significantly decreased the amount of cellulolytic bacteria and lactobacilli, whereas an augmentation of *Salmonella* and *C. difficile* was observed, compared to untreated horses. More recently, Álvarez-Narváez et al. [115] demonstrated the effect of oral erythromycin together with rifampin administered to horses suffering from subclinical pneumonia. Compared to a control group, a decrease of *Rhodococcus equi* and a general increase of ARGs copy numbers, suggesting an in-GIM selection of existing ARGs, were observed in treated horses. Similarly, Arnold et al. [116] reported a decrease of cecal and fecal microbiome diversity of five horses receiving metronidazole directly in the caecum; however, the results were not compared to those of an untreated control group. Another study showed that horses with diarrhea induced by an antibiotic treatment had an altered microbiota composition compared to horses not receiving antibiotics or those that did not develop post-antibiotic treatment diarrhea [117]. In horses, cellulolytic bacteria residing in the GIM seemed to be most affected by antibiotic treatments, undergoing a decrease in abundance. This decrease could negatively affect horses' health, considering that these bacteria are crucial to digest fibers, which are the main components of horses' diet.

7. Dogs and Cats

A catalog of the dogs' GIM is available and has unveiled a large similarity to that of humans with Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria as main phyla [118].

Pilla et al. [119] analyzed the effects of metronidazole, a broad spectrum antibiotic and anti-parasitic used to treat diarrhea, in the GIM of 1–10-years-old healthy dogs of different breeds, and in parallel the effect of changing diet. Metronidazole caused a decrease of Bacteroidetes and Fusobacteria and an increase of Proteobacteria and Actinobacteria. Abundance of Firmicutes remained constant but composition changed by diminishing Clostridiales and increasing Lactobacillales. At 42 days after antibiotic withdrawal, GIM composition returned to the baseline, except for the Fusobacteria abundance. This phylum in dogs is associated to good health status. Similar results for Fusobacteria and metronidazole administration were found by Igarashi et al. [120], who also reported an increase of Actinobacteria. When metronidazole combined with spiramycin was administered to diarrheic dogs, no difference was found in the amount of certain genera in the GIM compared to dogs administered with a nutraceutical compound [121].

Another commonly administered antibiotic to cure dogs' diarrhea is tylosin. Manchester et al. [122] observed a decrease of *Fusobacteriaceae* and *Veillonellaceae* and *Bacteroidaceae*, together with an increase of *Enterococcaceae*, after tylosin treatment. At tylosin withdrawal, return to the baseline was individual-dependent. Similarly, Suchodolsky et al. [123] reported long-lasting modification of dogs' GIM by tylosin treatment with a decrease of Fusobacteria, Bacteroidales, and *Moraxella*, parallel to an increase of enterococci, *Pasteurella* spp., and *Dietzia* spp.

Grønbold et al. [124] analyzed the effects of amoxicillin on dogs' GIM, which was enriched with Enterobacterales, with *E. coli* isolates exhibiting higher rate of resistance compared to isolates recovered at the pre-treatment sampling. A similar selection of resistant *E. coli* isolates was found by Werner et al. [125] in diarrheic dogs treated with amoxicillin-clavulanic acid. Amoxicillin and the amoxicillin-clavulanic acid combination affected in a similar way the species diversity and richness of treated dogs. Besides,

amoxicillin-clavulanic acid reduced gut commensal taxa, such as *Roseburia*, *Oscillospira*, *Dialister*, and *Lachnospiraceae* along with increase of *E. coli*. This drugs combination selected ampicillin-resistant *E. coli* and enterococci [126].

Longitudinal studies reporting the effects of antibiotics on cats' GIM are lacking. To the best of our knowledge, one report described the effect of clindamycin and showed, in clindamycin-treated cats, a decrease of Actinobacteria, Bacteroidetes, *Ruminococcaceae*, *Veillonellaceae*, and *Erysipelotrichaceae* along with an increase of *Clostridiaceae* and Proteobacteria [127] (Table 4).

Table 4. Summary of studies investigating impact of antibiotics on companion animals' intestinal microbiota and relative antibiotic resistance genes.

Host	Antibiotic	Farm	Administration	Dose (mg/kg of Animal)	Duration (Days)	Method	Effect on GIM	Effect on ARGs	Reference
Horses	Penicillin Ceftiofur Trimethoprim /sulfadiazine	ND	IMI O	20.000 UI/kg 2.2 30	5	16S rDNA (V4)	Diversity, richness, Verrucomicrobia ↓	ND	[7]
	Benzyl- Penicillin	ND	IMI	20.000 UI/kg	5	Cultivation, DGGE-16S rDNA (V3)	Bacteroidetes, Clostridiotes, Enterococcaceae ↑ Lactobacillaceae, cellulolytic bacteria ↓ <i>Salmonella</i> , <i>C. difficile</i> ↑	None	[113]
	Trimethoprim/ sulfadiazine Ceftiofur	ND	O IMI	30 2.2	7	Cultivation		ND	[114]
	Erythromycin Rifampicin	ND	O	30	14	16S rDNA (V4), shotgun, cultivation	Diversity, abundance, <i>Rhodococcus equi</i> ↓	ARGs macrolides, rifampin, doxycycline ↑	[115]
	Metronidazole	ND	O	30	3	16S rDNA (V4)	Diversity ↓	ND	[116]
	Multiple	ND	ND	Multiple	2–14	16S rDNA (V4)	Fusobacteria ↓ Tenericutes ↓ WPS-2* ↓	ND	[117]
Dogs	Metronidazole	ND	O	30	14	16S rDNA (V4), qPCR	Diversity, richness, Bacteroidetes, Fusobacteria, Clostridiales ↓ Proteobacteria, Actinobacteria, Lactobacillales ↑	ND	[119]
	Metronidazole, prednisolone	Exp	O	25/1	14	16S rDNA (V4)	Diversity, richness, Fusobacteria Clostridiales ↓ Actinobacteria, Bacilli ↑	ND	[120]
	Metronidazole/ spiramycin	ND	O	12.5/7.500 UI/kg	6	qPCR	None	ND	[121]
	Tylosin	ND	O	40	14	16S rDNA (V4), qPCR	Enterococcaceae ↑ Fusobacteriaceae, Veillonellaceae, Bacteroidaceae ↓	ND	[122]
	Tylosin	Exp	ND	20–22	14	16S rDNA (V4–V5)	Diversity, richness, Fusobacteria, Bacteroidales, <i>Moraxella</i> ↓ Enterococci, <i>Pasteurella</i> spp., <i>Dietzia</i> spp. ↑	ND	[123]
	Amoxicillin	Exp	O	20	7	DGGE-16S rDNA (V3), qPCR	Enterobacteriales ↑	AMX-resistant <i>E. coli</i> ↑	[124]
Amoxicillin/ clavulanic acid	ND	O	25–50	7	Cultivation, qPCR	None	AMX-resistant <i>E. coli</i> ↑	[125]	

Table 4. Cont.

Host	Antibiotic	Farm	Administration	Dose (mg/kg of Animal)	Duration (Days)	Method	Effect on GIM	Effect on ARGs	Reference
	Amoxicillin				5–13		Diversity, richness, Firmicutes ↓		
	Amoxicillin/clavulanic acid	ND	O	10–20	5–14	Cultivation, 16S rDNA (V3–V4)	Proteobacteria ↑ <i>Dialister</i> , <i>Oscillospira</i> , <i>Roseburia</i> , <i>Lachnospiraceae</i> ↓	AMX-resistant Enterococci and <i>E. coli</i> ↑	[126]
Cats	Clindamycin	Exp	O	12.1–22.7	21	16S rDNA (V4), qPCR	<i>Actinobacteria</i> , <i>Bacteroidetes</i> , <i>Ruminococcaceae</i> , <i>Veillonellaceae</i> , <i>Erysipelotrichaceae</i> ↓ <i>Clostridiaceae</i> , <i>Proteobacteria</i> ↑	ND	[127]

Note. GIM: gastro-intestinal microbiota; ARGs: antibiotic resistance genes; ND: not determined; IMI: intra-muscular injection; DGGE: Denaturing Gradient Gel Electrophoresis; O: oral; *: candidate phylum (Eremiobacterota); Exp: experimental; AMX: amoxicillin.

8. Conclusions

Overall, in all animal hosts and irrespective of antibiotic classes, a decrease of species diversity and richness was reported after treatment. In most cases, GIM composition demonstrated resilience, returning to baseline condition for composition and ARGs amount after treatments withdrawal. The time necessary to the baseline return was variable among studies.

At sub-therapeutic doses, certain genera belonging to the Firmicutes phylum and involved in sugar metabolism augmented in animals receiving antibiotics in food, suggesting a link with weight gain. This finding cannot be considered as an encouraging result, because usage of antibiotics even at low concentration is linked to the augmentation of ARGs in the intestine by selection and by triggering genetic transfer events with negative consequences for global health. The enhanced food-producing animals' performance obtained using antibiotics as growth promoters could be achieved by replacing antibiotics with modulation of the GIM with probiotics, for instance. For this reason, it is necessary to increase studies in the field, in order to unveil not only GIM composition but also metabolic processes assumed by taxa enriched during antibiotic administration.

At therapeutic doses, bloom of genera hosting potential pathogens was reported recurrently. The risk of this negative effect of antibiotic therapies is ineluctable. Besides, duration of the therapy and dosage could play a role in modulating the intensity of this side effect. In parallel to dysbiosis, selection of ARGs occurs, as well. Generally, selective action and consequences on GIM composition were lower for parenteral administration, but effects could still be detected. Indeed, those drugs with hepatic metabolism can reach the gut not-metabolized, together with their metabolites, through bile secretion [128]. Such effects, besides being drug-dependent, are also dependent on pharmacodynamics parameters proper to each animal species [129]. More studies are necessary to clarify these aspects in order to improve therapy duration, antibiotic dosages, and administration routes in the effort of mitigating negative effects of antibiotic therapies. In addition, this review highlights that studies on aminoglycosides are almost inexistent; this is a serious knowledge gap that should be filled considering that aminoglycosides represent the first most commonly used antibiotic class in companion animals worldwide.

Based on the current knowledge, it is difficult to choose an antibiotic, or an antibiotic class, that could have less negative effects compared to others and in the meantime serve as successful treatment. Comparison among studies is hindered by variations in the experimental design including drug concentration, antibiotic combination, therapeutic regimen, and duration of the treatment. Harmonization of experimental procedures is crucial, as well. In fact, studies analyzing animal GIM are often conducted by sequencing the V3–V4 region of the 16S rDNA, but not always. Each hypervariable region is more

specific for certain taxa, thus studies based on different hypervariable regions are difficult to compare. The advent of long reads-sequencing could overcome such difficulties. Long-reads sequencing will most likely also improve results generated by shotgun sequencing, improving assembly and prediction of gene function on taxonomic analysis. Shotgun metagenomics should be preferred for future investigations to gather comprehensive knowledge on GIM and ARGs with their genetic elements considering that in current longitudinal studies analyzing antibiotics effect on GIM, the ARGs analysis has been largely neglected.

This review, encompassing the main animal hosts and all antibiotic classes, provides inspiration for future investigations, highlighting the major knowledge gaps that need to be filled for improving antibiotic usage and mitigating negative effects of these drugs. Decorticating GIM composition and function will unveil revolutionary strategies for medication and improvement of animals' health status, resulting in positive consequences on global health.

Author Contributions: T.R.: Bibliography search, data curation, writing original draft, T.R.; Funding acquisition, conceptualization, reviewing, M.H.; Reviewing and editing, S.M.; Reviewing, C.A.; Reviewing and editing, G.C.; Funding acquisition and editing, T.F.; Funding acquisition, conceptualization, reviewing and editing, J.-Y.M.; Conceptualization, supervision, writing, reviewing and editing, A.L. All authors have read and agreed to the published version of the manuscript.

Funding: The PhD thesis of Tony Rochegüe was supported by the LABEX ECOFECT (ANR-11-LABX-0048) of Université de Lyon, within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR) and proper funding from ANSES.

Institutional Review Board Statement: No ethical approval was necessary for this manuscript.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available to be shared at any request.

Conflicts of Interest: The authors declare no conflict of interest.

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3. Analysis of *Escherichia coli* isolated from calves' stools before and after an amoxicillin therapy

The second axe of the project consisted in the analysis of Gram-negative cultivable bacteria from the stools of treated and untreated calves of this study. Enumeration of the bacteria on the selective and free medium from the three sampling times did not point a statistically significant difference between sampling times and between treated and untreated calves. Colonies were selected for a qualitative analysis including antibiotic susceptibility and genomic content characterization (genetic relatedness, antibiotic resistance genes, genetic elements, and virulence factors) by whole genome sequencing.

Isolates were all identified as *E. coli*. All amoxicillin resistant isolates harbored the *bla*_{TEM-1} gene associated with insertion sequences, such as IS26, and IS91, or with Tn3 transposon. The *bla*_{TEM} gene was located close to other ARGs and to class 1 integrons. Ten different class 1 integrons were found. *E. coli* isolates harbored several plasmids mostly of the IncFIA-IB, IncFII and IncQ1 type. Virulence factors found in the isolates were numerous and diverse, but no association was found with other features of the isolates, including sequence type, sampling time, farm of calf residence.

SNPs analysis of *E. coli* isolates belonging to the same sequence type demonstrated the presence of similar clones within the same calf and between calves residing in the same farm and even in independent farms, suggesting the role of potential vectors for the *E. coli* dissemination and presence of persistent reservoirs.

The manuscript presenting the results of this axe is in preparation.

Analysis of *Escherichia coli* isolates from stools of calves undergoing amoxicillin therapy.

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Abstract

Amoxicillin is used for the treatment of non-severe infections in both humans and animals. Amoxicillin therapies have been associated to the selection of resistant bacteria in numerous hosts. Longitudinal studies investigating resistance selection after amoxicillin therapy in calves, a major food-producing animal in France, are lacking.

Stools were collected from calves' (n = 12) before amoxicillin treatment (T0), at amoxicillin treatment withdrawal (T1), one week after T1 (T2). At the same pace, stools were collected from untreated calves (n = 5). Gram-negative bacteria susceptible or resistant to amoxicillin were enumerated. Selected isolates (n = 152) were analyzed for their susceptibility. Genomes were sequenced and analyzed for genetic relatedness, antibiotic resistance genes, genetic elements carrying ARGs, and virulence factors.

Amoxicillin-resistant Gram-negative bacteria raised after amoxicillin therapy in treated calves and decreased at T2. Obtained colonies were essentially *E. coli*. The *bla*_{TEM-1} gene was found in all amoxicillin-resistant isolates, located close to aminoglycosides and tetracycline resistance genes. Class 1 integrons were found in most of the isolates. Plasmids of the IncFII, IncFIA-IB, and IncQ1 type were the most common. Genetically related isolates occurred in calves residing in different farms, with similarity raising with decreasing of the geographical distance between farms. *E. coli* clones were able to persist in the same farm for long periods.

Amoxicillin favored the selection of amoxicillin-resistant *E. coli* and co-selection of other antibiotic classes. Dissemination of clones in the farm and between farms deserves further investigation to mitigate resistance dissemination and preserve the action of crucial antibiotics.

Key words: kanamycin, tetracycline, class 1 integrons, ST10, ST58, *bla*_{TEM}, IncFIA-IB, IncFII.

Introduction

Amoxicillin is one of the most used antibiotics worldwide, both in human and veterinary medicine. In France, amoxicillin has been the most used antibiotic both in hospital and community settings for the treatment of infections related to human obstetricians and gynecology during the 2012-2018 period. Amoxicillin consumption has raised compared to 2010 (+8%), in that time-lapse [1].

In veterinary medicine, amoxicillin belongs to the list of antibiotics to be used as first-line therapy to treat animals' skin and respiratory infections, among other conditions [2]. Amoxicillin can be administered by oral and parenteral administration and in humans is mostly excreted by kidney (80%) and in lower extent it is metabolized in the liver [3]. Thus, it is conceivable that amoxicillin can reach the intestine through bile excretion even after parenteral administration. The effect of amoxicillin therapy on the intestinal microbiota has been studied in humans [4, 5] and some animal species, including dogs [6, 7], pigs [8], minks [9], and chickens [10]. Overall, these studies reported an increase of determinants conferring resistance to amoxicillin, especially the *bla*_{TEM} gene, and amoxicillin resistant bacteria in the feces of treated hosts, together with a modification of the species composition of the bacterial population resident in the intestinal microbiota. Amoxicillin administration has been associated with the selection of resistance to other antibiotic classes [11, 12], as well. The excretion of antibiotic-resistant bacteria can favor the colonization of further hosts and nourish the transmission of resistance through the food chain [13].

In previous studies, effects of amoxicillin treatment in the selection of resistances have been proved by the cultivation of *Escherichia coli*. This species is the most abundant in the cultivable fraction of gut microbiota [14]. Emergence of antibiotic resistance in *E. coli* is a health concern. According to the report of RESAPATH, a surveillance system based on susceptibility data provided by a network of veterinary diagnostic laboratories [15], 85% of *E. coli* isolates from calves suffering from digestive tract infections are resistant to amoxicillin. In calves' gut, the occurrence of ESBL-producing *E. coli* is decreasing, however this major resistance mechanism warrant continuous surveillance [16].

In the light of this state of the art, selection of amoxicillin-resistant bacteria in the intestinal microbiota of calves by amoxicillin treatment is conceivable.

The effect of amoxicillin treatment on cultivable, aerobic, Gram-negative bacteria from calves' stools hosted in commercial farms was analyzed. Stools were sampled from calves' rectum before amoxicillin therapy, at amoxicillin therapy withdrawal, and one week after amoxicillin therapy withdrawal. At the same pace, stools were collected from untreated calves. Bacteria were cultivated and enumerated. Selected colonies were further analyzed by genome sequencing. ARGs and genetic elements supporting antibiotic resistance genes, genomic relatedness of isolates, sequence types, and eventual virulence factors encoding genes were described.

Material and methods

Calves features and fecal sampling

Fecal samples were prospectively collected from calves (n = 17) aged from five to 26 days, belonging to five breeds (Charolais/Montbeliard, Montbeliard, Prim' Holstein, Charolais/Prim' Holstein, Limousin/Montbeliard), and residents in seven farms (A-G) in the Rhône-Alpes region, France, during October 2018-March 2020. Twelve out of 16 calves were suffering from omphalitis, umbilical cord infection, and were treated with intramuscular injection of amoxicillin (Longamox, 15 mg/animal kg) for a duration varying between 4 and 16 days. The remaining five calves did not receive amoxicillin and their feces were sampled at the same time intervals of the treated ones. Three samples were collected from each treated calf: before amoxicillin treatment (T0); at the end of the treatment (T1); and one week after the end of the treatment (T2) (Table 1).

Feces were collected directly from the animal rectum, with a sterile glove and were conditioned and shipped to the laboratory according to the SOP n°3 of the IHMS consortium [17]. One gram of the feces was dissolved in 9 mL Brain Heart Infusion broth (Sigma, India) supplemented with 15% of glycerol and was dispatched in three cryotubes, each containing 1 mL, and kept at -80°C until further investigation.

Bacteria cultivation and enumeration

Seeding (50µL inoculum) from each cryotube was realized using the easySpiral Dilute (Interscience, France), with the method exponential on MacConkey agar plates (Sigma, USA) (90mm), prepared in house, without antibiotic and on MacConkey agar plates supplemented with amoxicillin, at a concentration selective for resistant *E. coli* (16mg/L) [18]. The selective power of each Mac Conkey batch was verified by seeding the amoxicillin susceptible CIP *E. coli* 76.24 on one plate. Lack of CIP *E. coli* 76.24 growth was interpreted as conformity. After

seeding, MacConkey plates were incubated overnight at 37°C, under aerobic condition. Colony-forming Units (CFUs) were counted using scan4000 v.7.0.10 (Interscience, France).

From one triplicate per fecal sample, the plate containing the highest number of separated colonies was chosen to isolate each colony morphologically differing from the others for further analysis.

In parallel, to search for ESBL producing bacteria, 100µL of the sample contained in the cryotubes was inoculated in BHI broth containing cefotaxime (2mg/L). After 24h incubation at 37°C with shaking, 100µL were seeded on MacConkey agar plates containing cefotaxime (2mg/L).

Colonies identification and antibiotic susceptibility testing

Isolated colonies were purified and identified by 16S rRNA mass spectrometry (MALDI-Toff, ANSES-Laboratory of Nancy). Isolates identified as *E. coli* were tested for their antibiotic susceptibility, to a panel of 30 antibiotics (amoxicillin, amoxicillin-clavulanic acid, piperacillin, piperacillin-tazobactam, cefalotine, cefuroxime, cefotaxime, ceftiofur, ceftazidime, cefoxitin, cefepime, cefquinome, aztreonam, ertapenem, tetracycline, kanamycin, tobramycin, gentamicin, amikacin, apramycin, netilmicin, streptomycin, florfenicol, chloramphenicol, colistin, sulfonamides, trimethoprim, nalidixic acid, enrofloxacin, and ofloxacin) by disc diffusion on Mueller–Hinton agar (Biorad, France), according to the guidelines of CA-SFM. Interpretation of inhibition diameters was based on veterinary CA-SFM breakpoints [19].

Molecular analysis

E. coli isolates were analyzed by multiple-locus variable-number tandem-repeat analysis (MLVA) [20]. From each calf, and sample time, one isolate with a unique MLVA or susceptibility profile was further analyzed by genome sequencing. To this end, DNA was extracted using the NucleoSpin Microbial DNA extraction kit (Macherey-Nagel, France) according to the manufacturer's protocol. Library preparation was performed using the Nextera XT technology, and sequencing was performed on a NovaSeq instrument (Illumina, San Diego, CA, United States) at the Institut du cerveau et de la moelle épinière (Paris, France) generating 150 bases paired-end reads. Quality of obtained reads was evaluated by FastQC v.0.11.9 [21]. Reads were quality trimmed using Trimmomatic v.0.39. Remaining reads were *de novo* assembled using Shovill v1.0.4, and the quality of assemblies was assessed using QUAST v5.0.2 on the platform Galaxy Australia (<https://usegalaxy.org.au/>). Annotation was performed using RAST v.2 on platform PATRIC [22, 23].

Genome analyses were performed using the Center for Genomic Epidemiology website (www.genomicepidemiology.org). In particular, Resfinder v.4.1 [24, 25] and Virulence finder v.2.0.3 [26] were used for ARGs and virulence factors content determination; sequence type (ST) was determined using MLST v.2.0.4 [27]; genetic elements and plasmids were searched using the MGE tool v.1.0.3 [28] and plasmidFinder v.2.0.1 [29], respectively; when opportune, SNPs variation among clones was analyzed using CSI phylogeny [30]. Relation of detected STs was evaluated using eBurst and the STs repertoire of the Achtman scheme (Accessed on 1/09/2021) [31].

Graphical representation of genetic elements was realized using Easyfig 2.2.5 [32].

Statistical analysis

Variation in the resulting standard deviation of CFU enumerations from the three aliquots of the fecal samples was calculated using Excel (Microsoft Office, 2016). Using the biostatTGV website, statistical significance ($p < 0,05$) of CFU variations from MacConkey and MacConkey supplemented with amoxicillin enumeration were evaluated comparing values from T1 to T0 and from T2 to T0 (Wilcoxon signed-rank test), and at each sampling time between treated and untreated calves (Wilcoxon Mann Whitney test).

Results and discussion

Bacterial count on Gram-negative selective medium with and without amoxicillin

Enumeration of CFU/g of feces suggested that the amount of living Gram-negative, lactose-fermentant and aerobic bacteria varied among calves before amoxicillin treatment (T0), with the lowest value calculated in calf 2 (2.47×10^5) and the highest in calf 12 (8.67×10^8). After amoxicillin treatment (T1), a decrease of the bacterial amount was observed in most of the treated calves (3, 4, 5, 9, 11, 12, 13), and in all untreated ones (16, 18, 19, 20). Variation of bacterial enumeration among sampling times was not statistically significant (Wilcoxon-Mann Whitney test, $p > 0.05$). In the untreated group, and some of the treated calves (3, 9, 14), a further decrease was observed at T2 (Figure 1). Such decrease of cultivable Gram-negative bacteria could be due to the maturation of calves' intestinal microbiota during the observation time. The maturation of the intestinal microbiota occurs around the first seven weeks of life and mostly consists in the augmentation of strictly anaerobic species at the expense of facultative anaerobic, lactose-fermenting species [33]. In general, taxa of the Proteobacteria and Firmicutes phyla are the first to colonize the neonatal intestine of

mammals, deriving from the mother, the colostrum, or more in general from the environment [34]. These species are supposed to create a favorable environment for anaerobic bacteria by consuming the oxygen present in the intestine [34].

The amount of bacteria enumerated on selective plates containing amoxicillin was similar to that enumerated on antibiotic-free medium, even before the amoxicillin treatment, with few exceptions (calf 16 at T0, and calf 19 and 20 at all sampling times) (Figure 1). No statistical differences in bacterial enumeration were observed between sampling times (Wilcoxon-Mann Whitney, $p > 0.05$) or between treated and untreated groups (Wilcoxon ranked test, $p > 0.05$). The observed high occurrence of amoxicillin-resistant bacteria in calves' feces is in accordance with the general epidemiological situation. In fact, *E. coli* isolates issued from calves are commonly resistant to amoxicillin (85%; $n = 4647$, in 2019), in France [35]. This high prevalence could be due to long-lasting historical usage of amoxicillin, and although a considerable decrease of penicillins usage has been observed for treating bovines in France since 2011, this class of antibiotic remains still the most consumed for treating bovines infections by parenteral administration [36]. A correlation between history of amoxicillin usage and the occurrence of resistant *E. coli* in calves has been reported [37]. Selective activity of ampicillin (which has similar activity compared to amoxicillin) on resistant bacteria, and respective resistance genes, has been analyzed also in a longitudinal study on pigs in which ampicillin treatment, especially by oral administration, significantly augmented the amount of ampicillin-resistant *E. coli* and *bla*_{TEM} gene in treated pigs compared to untreated ones [38]. In the current study, the lack of statistical significance in the amount of amoxicillin-resistant bacteria enumerated after treatment compared to pre-treatment or control group could be due to several additional factors. In particular, at the pre-treatment variation of bacteria amount among calves could affect the statistical resolution of the analysis. According to breeders' declaration, calves were breastfed and mothers were not known to receive antibiotic therapies, thus selection of resistance by other factors linked to nutrition could be mitigated.

Overall, selective power of antibiotics at therapeutic concentration occurs, even when the administration route is not oral. Amoxicillin is only in part metabolized in the liver and excreted in the bile, which in turn is excreted in the intestine where selection of bacteria can occur according to the concentration of the antibiotic [39]. However, differences in the metabolism of amoxicillin between animals could exist, and to the best of our knowledge metabolism of amoxicillin has not been investigated in calves.

Calves of the current study were not colonized with ESBL-producing bacteria.

Clones and genetic relatedness of *Escherichia coli* isolates

To evaluate qualitatively the effect of amoxicillin therapy on the intestinal bacteria, *E. coli* isolates were purified and their genomes sequenced. This species was targeted because is the most abundant in the cultivable fraction of the intestinal microbiota [40] and also for its valuable function as a bacterial indicator of clinically relevant resistances for both animals and humans [41]. After excluding those colonies retrieved from the same plate and presenting identical MLVA (data not shown) and antibiotic susceptibility profile, 152 isolates were further analyzed by NGS.

Among the 152 isolates, 41 STs were represented. An eBURST analysis including STs of the current study together with the repertoire of *E. coli* STs evidenced that all STs, except ST3042, were related to other STs or belonged to previously defined clonal complexes (CCs) (Figure S1). The most represented was CC10 to which ST10/34/48/167 belonged to, followed by CC23 (ST23 and ST88), and CC155 (ST56 and ST58). Other clonal complexes (CC20/29/69/ 31/165/278) occurred with one representing ST.

Isolates belonging to CC10 have been previously isolated from calves [42] and other hosts [43], with increasing evidence as a major etiological agent of invasive infections also in humans [44]. Previous reports described that genomes belonging to CC10 clustered in multiple sub-lineages not according to host, or geographical origins. Acquisition of genetic elements by horizontal gene transfer is largely contributing to the evolution of CC10 [45]. In the recent past, isolates of the CC23 have been reported as Extended-spectrum AmpC beta-lactamases producers from both animals [46] and humans [47], in France. Besides, CC23 is undergoing to global dissemination [48-50], similarly to CC155 [51, 52] and other CCs [53] were encountered in the current study. Globally, isolates collected in calves of this study reflected common epidemiology of the *E. coli* population, with clones at high potential for dissemination.

Mostly, isolates belonging to the same clonal complex belonged to the same phylogroup as defined by Clermont et al. [54]. Isolates of CC10, CC23, and CC165 belonged to phylogroup A, whereas isolates of CC155 belonged to phylogroup B1. Isolates of CC69 belonged both to phylogroup D and B1. Globally, phylogroup A was the most represented in the collection (n = 57/152), followed by phylogroup B1 (n = 54/152), phylogroup D (n = 38/152), and B2 (n = 3/152). This distribution reflects the analysis on the *E. coli* genomes from Enterobase, that highlighted B1 and A phylogroups as the most prevalent [55].

Analyzing occurrence of STs according to sampling, at the pretreatment (T0) 27 STs occurred, 21 at amoxicillin withdrawal (T1), and 30 at T2 (one week after amoxicillin withdrawal). Certain STs occurred in more than one calf resident in the same farm. At T0, ST58 was the most diffused occurring in calves resident in five (A, B, D, F, G) out of seven farms. ST58 was followed by ST10, found in calves from farms A, B, D, and F. ST117 was found in calves resident in farms A, B, and E, whereas ST48 and ST362 occurred in calves from two farms (A and B, D and E, respectively). Thirteen STs (STs: 10, 34, 48, 56, 58, 69, 117, 164, 362, 949, 1302, 6118, 11887) were detected at T0, occurred also at amoxicillin withdrawal (T1), and one week after amoxicillin withdrawal (T2). ST23 and ST950 were detected only at T0 and T1. Several STs occurred at T0 and re-emerged at T2 (STs: 130, 167, 301, 540, 659, and 2325) (Figure 2).

For all the isolates belonging to the same ST, genetic relatedness was further analyzed according to Single Nucleotide Polymorphisms (SNPs) occurring in their genomes. Some of these isolates occurred in a unique calf at different samplings. In several cases, these isolates were very close to each other with SNPs ranging from 0 to 44. To understand how amoxicillin therapy could have influenced the emergence of such SNPs, further analysis to unveil the genetic determinant involved in the polymorphisms needs to be performed. However, several isolates belonging to the same ST and from the same calf presented numerous SNPs (295-24141) suggesting a high degree of divergence (Table 2).

Isolates presenting a small number of SNPs and originating from different calves were found, as well. Often, calves colonized with the most similar clones were resident in the same farm and were sampled in the same period (Table 3). However, calves residing in the same farm at different times, in 2018 and 2020, were found colonized with *E. coli* clones close to each other. This was the case of calves 2 and 19 from farm A, colonized with 55270 and 54112, respectively, presenting 21 SNPs. Similarly, isolates 54108 (year 2020) and 55260 (year 2018) were isolated from calves 20 and 2, respectively, from farm A and presented 16 SNPs.

Similarity, at a lower level, was found also among isolates collected from calves residing in different farms for instance isolates from farms A, C, and E presented 21 SNPs, isolates 57963 and 55401 from farm G and C 29 SNPs. Similarity decreased for isolates from other farms (31 SNPs between isolates 55285 and 55475 from farm B and E, 55 SNPs between isolates 55269 and 55342 from farm A and B, 60 SNPs were found among isolates from farm D and E, and 61SNPs were detected between 55268 and 54161 from farm A and E (Table 3).

Previous studies have reported SNPs analysis in *E. coli* clones evolving in the host, highlighting the emergence of 5 or 10 SNPs between subsequently isolated strains during the observation period [56]. The highest number of SNPs in the isolates could be attributed to recombination events mediated by phages. Another report has highlighted the occurrence of 8 SNPs in *E. coli* clones sub-sequentially isolated from the same host [57]. Other reports have interpreted the occurrence of less than 40 SNPs as close genetic relatedness among isolates [58]. In most cases, isolates of the current study found in the same calf before and after amoxicillin treatment presented less than 10 SNPs suggesting the persistence of the same clone in the host after amoxicillin treatment. Interpreting genetic relatedness for those isolates presenting more than 10 SNPs is less evident. A clonal relatedness among these isolates cannot be excluded, with variations attributable to recombination events. However, further colonization with other related clones during treatment cannot be excluded, as well. The presence of isolates differing for less than 40 SNPs and found in calves residing in the same farm but in different years suggest the persistence of the same clone in the environment and propagation to further hosts during the time. To unveil the determinants of such persistence a global analysis of *E. coli* population of adult bovines, farmers, food and drinking water of the farm, along with environmental supports is need. Genetically close isolates (SNPs < 40) have been found in calves residing in different farms. The smallest amount of SNPs (n = 21) was detected among isolates from farms A, C and E. These farms were located at 4 and 9 km from each other. Farms B and E (relative isolates SNPs = 31) were 6.5 km apart, similarly to farms G and C (5.7 km) whose relative isolates differed for 29 SNPs. Farms D and E were 22 km apart and relative isolates differed for 60 SNPs. Farm A and B were 28.5 km from each other and isolates found in calves of these farms differed for 55 SNPs. Overall, this analysis suggested that calves resident in farms in the range of 10 km had a higher probability to be colonized with similar clones. Among other, elements that could bacteriologically connect farms located in the same geographic perimeter are water, rodents, and insects. Domestic flies, for instance, can cover a perimeter flight as large as 7km. The role of coprophagic insects in the dissemination of antibiotic resistance is gaining increasing interest and they should be taken in account when analyzing dissemination of resistance in contexts like farms, where feces are exposed to insects for long periods [59, 60].

Antibiotic resistances and genetic elements

Before amoxicillin treatment (T0) 54 *E. coli* were isolated, 47 at amoxicillin withdrawal (T1), 51 one week after T1 (T2). More than one colony per sample was further

analyzed, thus an exact quantification of resistances at the three samplings is hindered. However, augmentation of amoxicillin-resistant *E. coli* (from 69% to 81%) was observed at T1 and persisted at T2 (77%) (Figure S2). This observation is in accordance with the quantification of the *bla*_{TEM} gene obtained by qPCR. A four-fold increase of the *bla*_{TEM} gene, relatively to the 16S rRNA gene occurred from T0 to T1. Similarly, the *tetA*, *strA/B* and *aph3'-Ia* genes, responsible for tetracycline, streptomycin and kanamycin resistance, increased after amoxicillin treatment by qPCR analysis (submitted data). These results were in agreement with the raising of tetracycline, kanamycin and streptomycin-resistant *E. coli* proportions at T1. At T1, also more chloramphenicol and fluoroquinolones resistant isolates were found (Figure S3).

In the majority of the amoxicillin-resistant isolates (n = 103/114, 90%) the gene *bla*_{TEM-1B} was present. The remaining amoxicillin-resistant isolates (n = 11/114, 10%) harbored the variant *bla*_{TEM-1A}. The spectrum of resistance conferred by these two variants is comparable, but the *bla*_{TEM-1B} variant is the most diffused [61]. This successful dissemination is probably due to the localization on transferable plasmids [62, 63] where it localizes close to *bla*_{CMY} or *bla*_{CTX-M} genes conferring resistance to third-generation cephalosporins. This situation could favor the co-selection [64] of more recent and with a wider beta-lactamase activity spectrum by amoxicillin.

In certain cases, it was possible to examine the genetic environment of the resistance genes detected in this study. In isolate 55263, *bla*_{TEM-1A} was located downstream a phage site-specific serine recombinase and upstream from a mercuric ions resistance (*mer*) operon. Upstream *bla*_{TEM-1A}, a copy of the *aph3'-Ia* gene, surrounded by two copies of the IS26, was present. According to a BLASTn analysis, this module was located on plasmids found in several Enterobacterales, but also in *Acinetobacter* spp. In 55291 and 55293 isolates, the *bla*_{TEM-1A} gene was located close to a *mer* operon, as well.

In four isolates (55351, 55296, 55294, 55315), *bla*_{TEM-1B} was located close to genes encoding for a type-four secretion system, involved in conjugative machinery construction.

In the majority of amoxicillin-resistant isolates (n = 64), *bla*_{TEM-1B} was found downstream a copy of IS91 insertion sequence, and upstream a Tn3 resolvase encoding gene. In certain isolates (55470, 55458, 55432, 54144, 55436, 55429, 54101) the contig containing the *bla*_{TEM-1B} sequence, harbored also a part of Tn3. In isolate 55262, *bla*_{TEM-1B} was located on a Tn3 transposon together with other resistance genes (*strA*, *strB*, *sul2*, *dfrA14*). This element was found in further four isolates (54109, 54125, 54134, 54138). These genetic assets, besides

favoring the intra- and inter-cellular dissemination of antibiotic resistance genes, poses the basis for the co-selection of multiple classes of antibiotics. In isolates 55302, 55277, 55290, and 55340 the *bla*_{TEM-1B} gene was located close to a partial *mer* operon (Figure 3a). Origin of the *mer* operon was found in thermophilic Bacteria and Archaea. Proteobacteria have acquired the operon more recently. Beyond detoxification from mercuric ions contaminating water and soil, the *mer* operon contributes to detoxification from mercury constituting disinfectants, herbicides and pesticides [65], thus probably contributing to its selection and maintenance in bacterial species adapted to mammals. This could explain the occurrence of the *mer* operon in *E. coli* of bovines' intestine.

Often the *mer* operons were located close to class 1 integrons. Integrons are platforms capturing antibiotic resistance genes close to each other, favoring the development of multidrug resistance [66]. Ten different compositions of class 1 integrons were observed in this study, spanning from basal integrons (*intI1* gene followed by a resistance cassette) to complex integrons containing the *intI1* gene, resistance cassettes, and the *qacEΔ-sul1* region (Figure 3b). Transposons-related genes often surrounded the detected integrons, thus their mobilization is conceivable to occur [67]. A BLASTn analysis of the contigs containing the integrons suggested that they were found in the genome of several Enterobacterales but also in *Aeromonas hydrophyla* sbsp. *hydrophyla*, and *Morganella morganii*. Often, *E. coli* harboring integrons similar to those of the current study were associated with urinary tract infections in humans [68]. They were present in isolates from food-producing animals, as well [69].

Overall, class 1 integrons harbored the most of non-beta lactams resistance genes including *dfrA*, *sul*, *ant(3'')-I* and *ant(2'')-I* genes. One isolate possessed the *bla*_{OXA-1} gene, located in a complex class 1 integron (Figure 3b). Two isolates harbored a class 2 integron containing a *dfrA1* gene.

In numerous isolates (n = 13) the *tetA* gene, and its regulator encoding gene *tetR*, was located on a *Tn3* transposon, which was adjacent to a class one transposon (*intI1-dfrA5*).

In 73 isolates, *sul2* and *strA/B* genes were found located close to genes proper to plasmid replication belonging to the IncQ1 type. This type of plasmid was present in further eight isolates, but no resistance genes were co-localized with genes encoding for its structures. IncQ1 plasmids have been reported from a wide range of Enterobacterales genera and as carriers of genes representing major clinical concern for humans, such as *bla*_{KPC-2}, *bla*_{VIM-4} [70, 71]. Furthermore, an IncQ1 plasmid was found in a multidrug-resistant *E. coli* strain isolated

from a cat in France [72]. It appears that this plasmid with its large host range [73] and ability to capture resistance genes that confer resistance to multiple classes of antibiotics could contribute to the propagation of antibiotic resistance, importantly [74]. In the isolates of the current study, it is not excluded that other resistance genes could be co-localized with *sul2* and *strA/B* genes, but short-reads sequencing could not further elucidate the genetic organization.

The localization of these genetic elements on plasmids cannot be excluded. Further experiments are in progress to determine the plasmidic or chromosomal localization of these elements. Nevertheless, plasmids were common in this *E. coli* collection, with the most common belonging to the IncFII type (n = 48), followed by IncFIA-IB (n = 31), and IncB/O/K/Z (n = 30).

Finally, isolates demonstrating fluoroquinolones resistance did not harbor acquired fluoroquinolones resistance genes. In these isolates, typical mutations in the quinolone resistance determining region (GyrA, S83L, D87N/Y; ParC, S80I, E84G) conferring fluoroquinolones resistance occurred.

Virulence factors analysis

E. coli isolates of the current study harbored multiple virulence factors involved in adhesion, toxins and siderophores production, capsule/protectin synthesis, and colicin and type 3 secretion system (T3SS). Among the 152 isolates, only four did not harbor virulence factors.

Certain adhesins encoding genes occurred in most of isolates (*hra*, n = 97; 63.8%; *lpfA*, n = 87; 63.8%; *papC*, n = 77; 50.6%; *afa* operon, n = 68; 44.7%; *papA*, n = 65, 42.7%; *iha*, n = 64, 42.1%), whereas other were less common (*air*, n = 21, 13.8%; *eae*, n = 11; 7.2%; *efa1*, n = 6; 3.9%; *yfcV*, n = 3, 1.9%; *tsh*, n = 2, 1.3%) and *focC*, *sfaS/D* occurred in one isolate.

Five siderophores encoding genes occurred in the isolates of the current study including *iutA* (n = 102, 67.1%), *iucC* (n = 101, 66.4%), *fuyA* (n = 84, 55.2%), *ireA* (n = 62, 40.7%), and *iroN* (n = 37; 24,3%).

Toxins encoding genes were common among the isolates (*irp2*;n=69, 45.9%), *hlyF* (n=37; 24,3%), *astA*(n=27; 17,7%), *cdtB*(n=15; 9,8%), *ehxA*(n=7; 4,6%), *cnf1*(n=2; 1,3%), and *stx* (n=12; 7,8%). The shiga toxin encoding gene *stx1* was found in all the isolates

belonging to ST301/ 342/ 950 and 11887, whereas *stx2* was found in isolates belonging to ST659.

The capsule encoding virulence genes, *kpsE* and *kpsM*, were found in isolates belonging to several STs (130, 141, 69, 362, 11674) representing 13% of the isolates (n = 20). The *ompT* gene was found in most of the isolates (n=104; 68,4%).

Genes encoding for factors favoring the bacterial survival were found, as well, and included *iss* (n=110; 72,3%), which increases the survival in the serum, *traT* (n=116; 76,3%), involved in resistance to the immune complement, *eilA* (n=21; 13,8%), encoding a toxin promoter, *etsC* (n=37; 24,3%), encoding a secretion transporter, and *sitA* (n=93; 61,1%), encoding an iron transporter.

Colicins included multiple variants 1A (n=32; 21%), 1B (n=24; 6,3%), B (n=9; 5,9%), E1(n=17; 11,1%), E2 (n=20; 13,1%), H (n=52; 34,2%).

Genes encoding for T3SS were found in few isolates (n = 11) belonging to multiple STs (10, 20, 21, 301, 342 and 659). However, only a part of isolates belonging to ST10 (n = 2/12, 17%) harbored the T3SS encoding genes.

No relevant association was found between virulence factors encoding genes with the origin of isolates, in terms of farm hosting calves, sampling time, or STs. However, several STs found in the current study (10; 69; 167; 55; 88; 117 and 23) have been previously reported from isolates causing extra-intestinal infections [75].

Conclusion

Amoxicillin resistant *E. coli* occurred in all the calves enrolled in the study, even before amoxicillin treatment. This finding reflects the global epidemiology of amoxicillin resistant *E. coli*, which is globally disseminated. Genetically close isolates, presenting a reduced number of SNPs in their genomes, were found in calves residing in the same farm two years after the detection of the first clone, thus persistence of clones in the farm environment is conceivable to occur. Furthermore, the similarity of clones found in calves residing in different farms increased with decreasing of farms distances, suggesting the possibility of dissemination through a mobile vector between farms. Besides, a common source of contamination for calves living in different farms could be water, or purchased food, for instance.

All amoxicillin resistant isolates harbored the *bla*_{TEM-1A/B} gene, which were located close or into a *mer* operon or class1 integron. The structures of class1 integrons found in this

study were previously reported in other Enterobacterales genera, often located on a plasmid of different types, thus suggesting a possible mobilization of the integrons.

Although several virulence encoding factors were present in the isolates, no particular patterns could be revealed. Calves did not suffer from digestive pathologies, indeed.

Persistence of clones in calves' gut microbiota and in the farm environment seems to be driven by several factors further than antibiotic selective pressure. Old antibiotics can favor the development of multidrug resistance by the co-selective process. In the veterinary context, where the usage of antibiotics such as last generation cephalosporines, carbapenems and fluoroquinolones is forbidden, the occurrence of simultaneous resistance to amoxicillin, tetracycline, and certain aminoglycosides (streptomycin and kanamycin) strongly deplete the therapeutic arsenal for treating infections.

Whole-genome sequencing will probably shed light on the epidemiology and on the genetic supports of neglected genes, likewise *bla*_{TEM}, which still play a role in the development of resistance providing co-selection of resistance to other antibiotic classes and more recent beta-lactams. Investigation of environmental factors is crucial to understand bacteria circulation and host colonization in the farm system.

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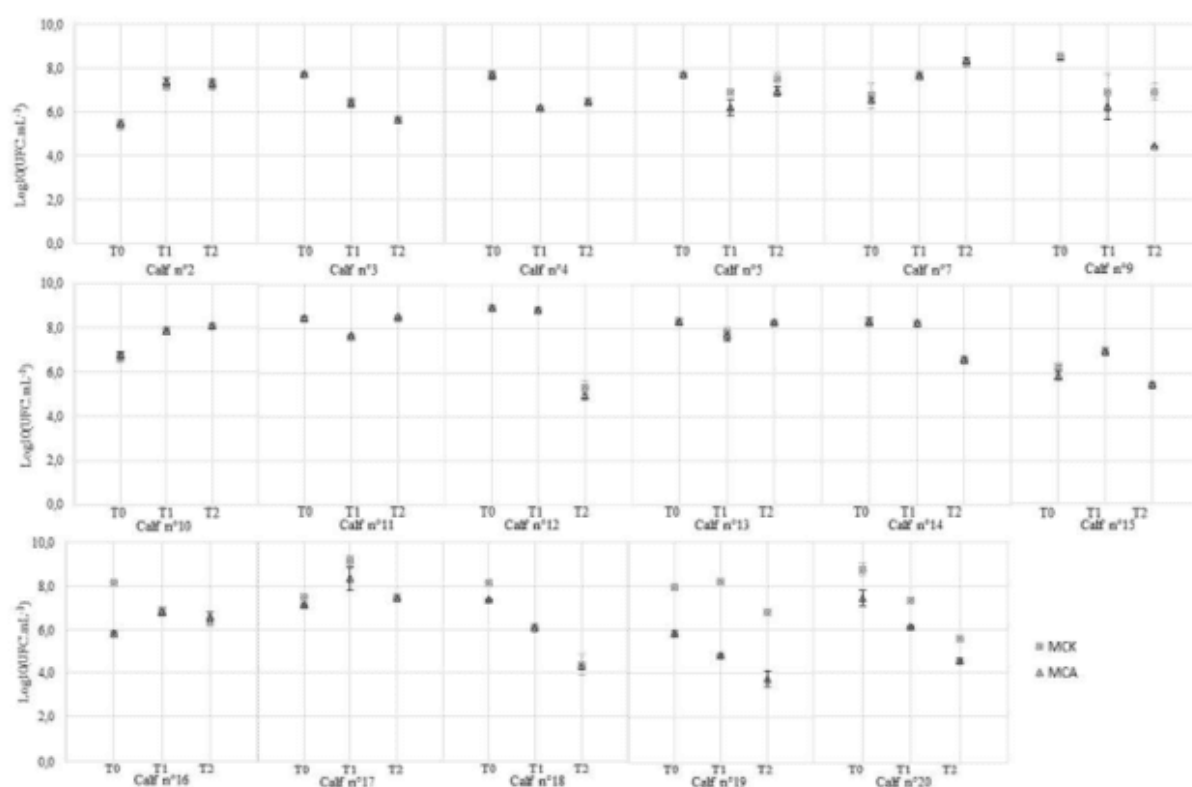


Figure 1. Colonies enumeration from calves' feces on MacConkey selective medium (MCK) and MacConkey containing amoxicillin (16 mg/L) (MCA) at three samplings: before amoxicillin treatment (T0), at amoxicillin withdrawal (T1), and one week after T1 (T2). Calf 16, 17, 18, 19, 20 did not receive amoxicillin treatment.

T0

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Figure 2. Occurrence of sequence types (ST) of *Escherichia coli* isolates found in calves feces at tree samplings, before amoxicillin treatment (T0), at amoxicillin withdrawal (T1), and one week after T1 (T2), and relative farm of residence.

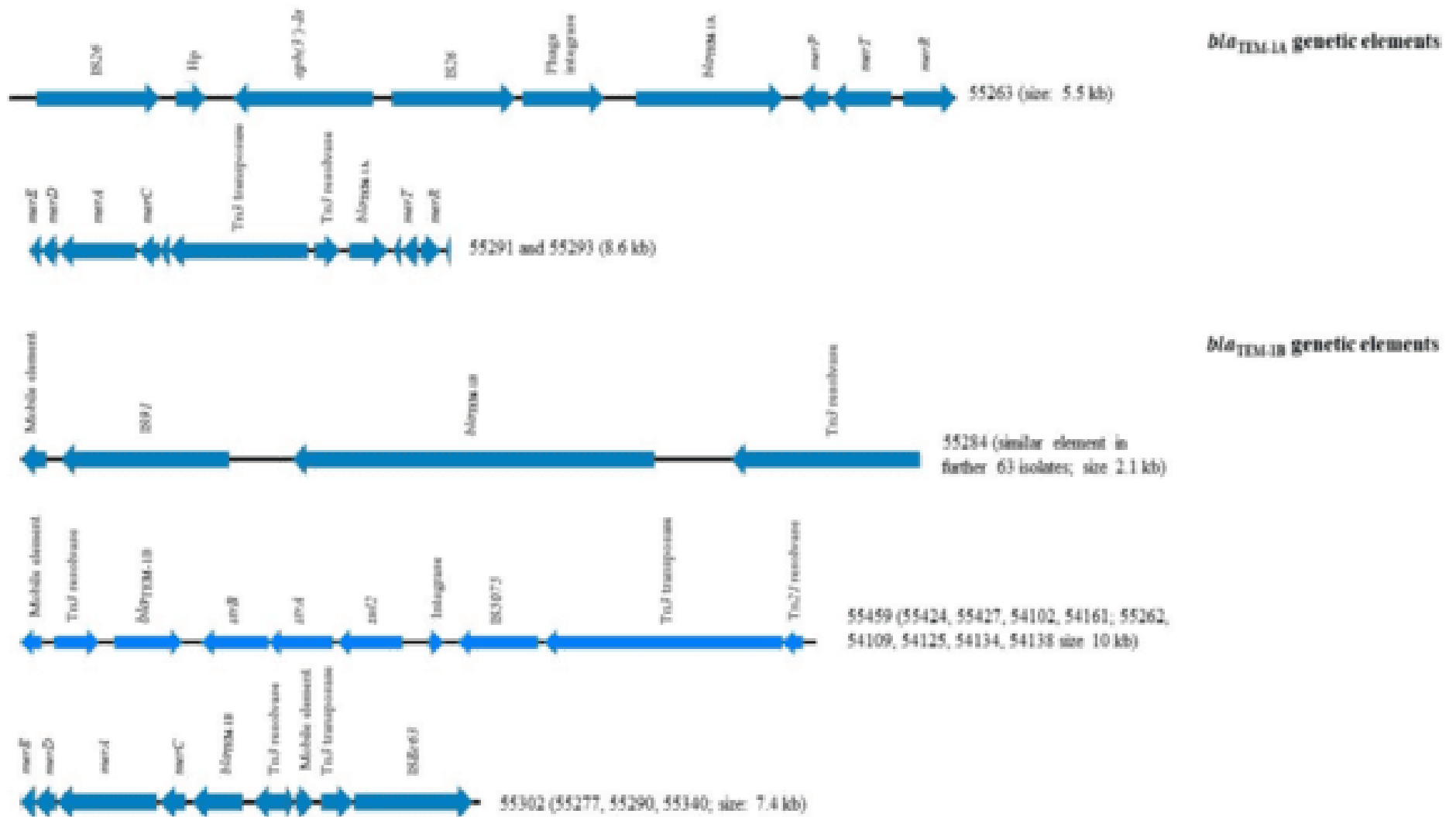


Figure 3a. Representation of *bla*_{TEM} genetic environment

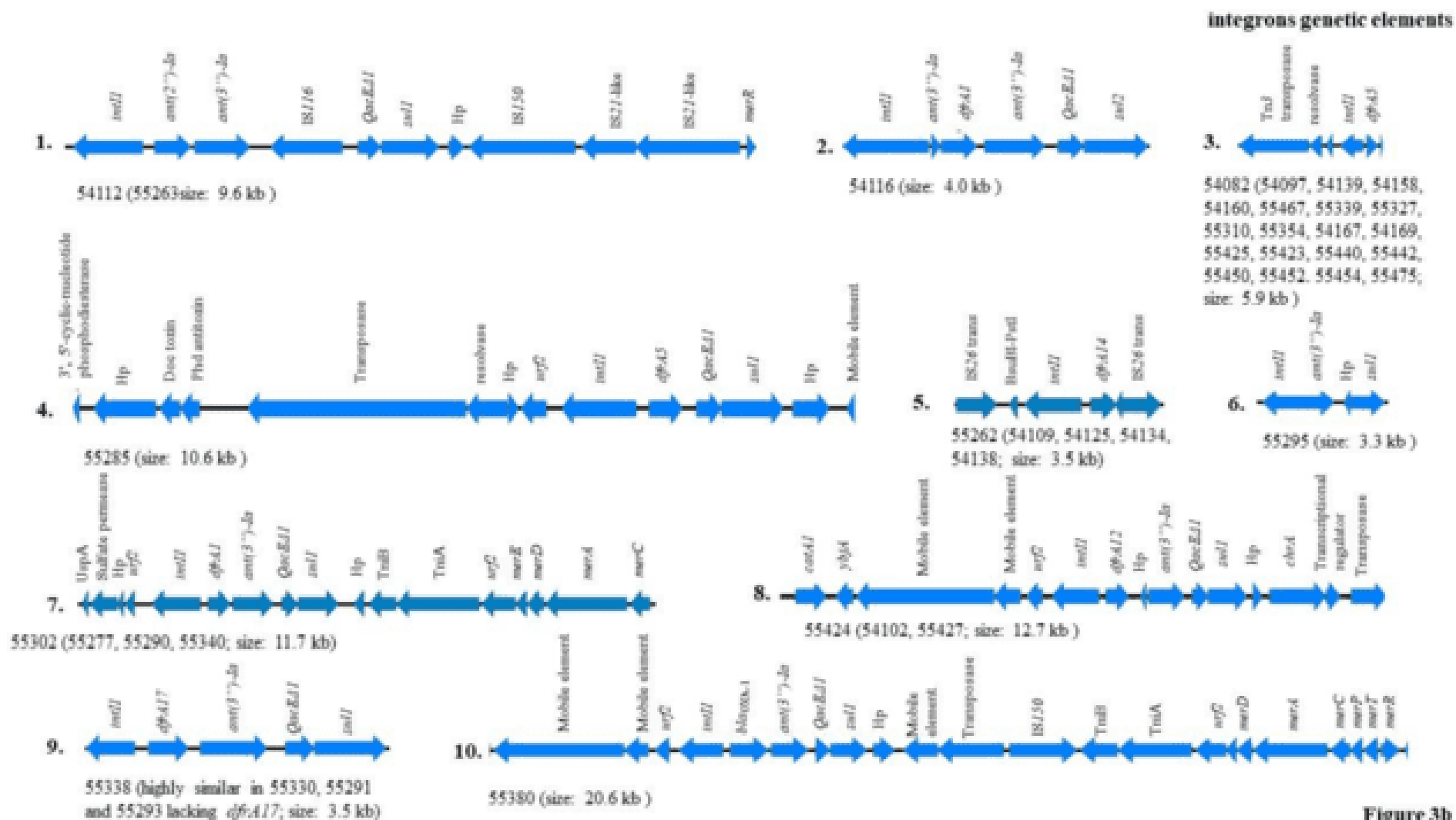


Figure 3b

Figure 3b. Representation of integrons class I and their cassette genes content

Table 1. Features of calves enrolled in the study and details of the amoxicillin treatment.

Calf information					Amoxicillin treatment				
ID	Farm	Breed	Sex	Age (days)	Start (T0)	End (T1)	7 days post T1 (T2)	Number of injections	Volume of antibiotic (15 mg/animal Kg)
2	A	Charolais/ Montbeliard	F	8 d	07/10/18	14/10/18	21/10/18	4	6 mL
3	B	Charolais/ Montbeliard	M	8 d	08/10/18	29/10/18	05/11/18	8	6 mL
4	B	Montbeliard	M	6 d	12/10/18	29/10/18	05/11/18	8	6 mL
5	B	Montbeliard	F	5 d	12/10/18	29/10/18	06/11/18	8	6 mL
7	C	Charolais/ Montbeliard	M	18 d	13/06/19	18/06/19	25/06/19	3	5 mL
9	C	Prim' Holstein	M	13 d	13/06/19	18/06/19	25/06/19	3	5 mL
10	C	Charolais/ Prim' Holstein	F	7 d	13/06/19	18/06/19	25/06/19	3	5 mL
11	D	Limousin/ Montbeliard	M	7 d	30/07/19	08/08/19	15/08/19	4	4 mL
12	D	Charolais/ Montbeliard	M	6 d	30/07/19	08/08/19	15/08/19	4	4 mL
13	D	Charolais/ Montbeliard	F	5 d	30/07/19	08/08/19	15/08/19	4	4 mL
14	E	Prim' Holstein	F	10 d	30/07/19	02/08/19	09/08/19	2	5 mL
15	F	Prim' Holstein	F	26 d	05/08/19	12/08/19	19/08/19	4	5 mL
16	C	Montbeliard	F	8 d	13/06/19	18/06/19	25/06/19	-	-
17	E	Prim' Holstein	M	5 d	30/07/19	02/08/19	09/08/19	-	-
18	G	Montbeliard	M	7 d	06/03/20	13/03/20	20/03/20	-	-
19	A	Montbeliard	F	8 d	06/03/20	13/03/20	20/03/20	-	-
20	A	Charolais/ Montbeliard	M	7 d	06/03/20	13/03/20	20/03/20	-	-

Table 2. Analysis of single nucleotide polymorphism in isolate belonging to the same sequence type and found in a unique calf at different samplings.

Isolate			SNPs			ST	Calf	Farm
T0	T1	T2	T0-T1	T1-T2	T0-T2			
55285		55300			4959	10	3	B
54165	54168	54174	6	7690	7696		15	F
	55288	55299		23		21	3	B
55474	55477		12			23	14	E
54143	54149		31				17	E
55277	55290		5			48	3	B
55340		55356			4869		5	B
54122	54132	54136	13	14	7		20	A
	55294	55296		8		58	3	B
55327	55315		13				4	B
55339	55354	55347	295	296	246		5	B
55352		55334			19674		5	B
	55440	55442		5			12	D
55450	55452	55454	4	9	11		13	D
	55467	54160		1			14	E
54164	54169		3849				15	F
54082		54097			5		18	G
54125	54134	54138	3	4	3		20	A
55444	55447		16			69	12	D
55459	55451		9				13	D
55263	55267		39			117	2	A
55377		55381			44940		7	C
	55414	55407		3			10	C
55471	55466		0				14	E
55432	55429	55436	13	7	18	362	11	D
	55439	54101		44			12	D
55470	55538		42				14	E
57962	55388	55379	24067	24141	15780	949	7	C
55400	55404		29				9	C
55411	55412	55417	31	35	12		10	C
55291	55293		6			950	3	B
54142		54155			1478	2325	17	E
54085	54180	54098	14	9	5	11887	18	G

Table 3. Analysis of single nucleotide polymorphism in *Escherichia coli* isolates from different calves.

ST	Isolate	Calf	Farm	Average SNPs	Min	Max	Date of isolation
10	55285	3	B	31	31	31	8/10/2018
	55475	14	E				2/08/2019
	55300	3	B	9	9	9	5/11/2018
	55303	4					5/11/2018
34	55341	3	B	5	5	5	12/10/2018
	55301	5					5/11/2018
48	54108	20	A	16	16	16	6/03/2020
	55260	2					7/10/2018
	55302	4	B	19	16	22	12/10/2018
	55277	3					8/10/2018
	55290	3					29/10/2018
	55340	5					12/10/2018
56	55270	2	A	21	21	21	21/10/2018
	54112	19					13/03/2020
58	54109	19	A	3	2	5	6/03/2020
	55262	2					7/10/2018
	54125	20					6/03/2020
	54134	20					13/03/2020
	54138	20					20/03/2020
	55347	5	B	179	7	303	6/11/2018
	55315	4					29/10/2018
	55354	5					29/10/2018
	55327	4					12/10/2018
	55294	3					29/10/2018
	55296	3					5/11/2018
	55339	5					12/10/2018
	55440	12	D	5	1	12	8/08/2019
	55423	11					15/08/2019
	55454	13					15/08/2019
	55450	13					30/07/2019
	55442	12					15/08/2019
	55452	13					08/08/2019
	54160	14	E	1	1	1	09/08/2019
	54158	17					09/08/2019
	55423	11	D	60	56	66	15/08/2019
	54158	17	E				09/08/2019
	54160	14	E				09/08/2019

	55440	12	D				08/08/2019
	55442	12	D				15/08/2019
	55450	13	D				30/07/2019
	55452	13	D				08/08/2019
	55454	13	D				15/08/2019
	55467	14	E				02/08/2019
69	55451	13	D	12	3	18	08/08/2019
	55447	12					08/08/2019
	55433	11					30/07/2019
	55459	13					30/07/2019
	55444	12					30/07/2019
	55433	11					30/07/2019
117	55263	2	A	21	4	39	7/10/2018
	55267	2					14/10/2018
	55407	10	C				25/06/2019
	55414	10					18/06/2019
	55479	16					18/06/2019
	55466	4	E				2/08/2019
	54144	17					30/07/2019
	55471	14					30/07/2019
130	55264	2	A	225	225	225	7/10/2018
	54099	18	G				20/03/2020
164	57963	7	G	29	29	29	25/06/2019
	55401	9	C				18/06/2019
301	55269	2	A	55	55	55	21/10/2018
	55342	5	B				12/10/2018
342	55484	16	C	7	7	7	25/06/2019
	55389	7					18/06/2019
362	55458	13	D	18	7	48	30/07/2019
	55436	11					15/08/2019
	55432	11					30/07/2019
	55439	12					8/08/2019
	55429	11					8/08/2019
	54101	12					15/08/2019
540	55268	2	A	61	61	61	21/10/2018
	54161	14	E				9/08/2019
659	55410	10	C	25	25	25	13/06/2019
	55483	16					25/06/2019
949	57962	7	C	20	5	34	13/06/2019
	55404	9					18/06/2019
	55400	9					13/06/2019
	55411	10					13/06/2019
	55412	10					18/06/2019
	55482	16					18/06/2019
1011	54102	12	D	40	40	40	15/08/2019
	55427	11					8/08/2019
1302	54119	19	A	17	17	17	20/03/2020
	54123	20					6/03/2020
6118	55287	3	B	4	4	4	20/10/2018
	55305	4					5/11/2018

Note. Grey shadow distinguishes groups of similarities.

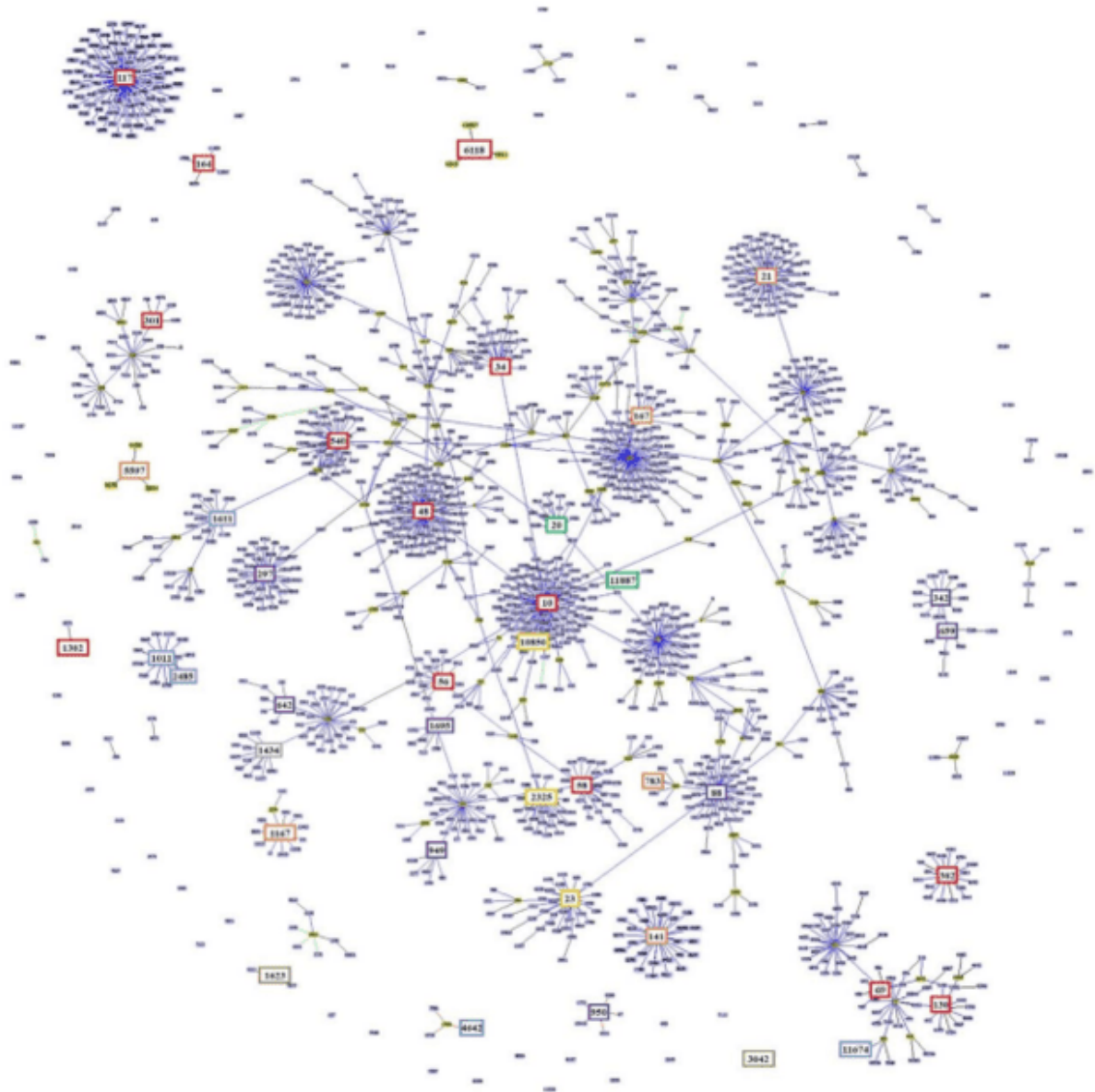


Figure S1. eBURST representation of *E. coli* sequence types (STs) including those found in calves' feces of the current study. STs found in the current study are highlighted according to farm residence of the calves (A: grey; B: orange; C: violet; D: blue; E: yellow; F: brown; G: green; multiple : red).

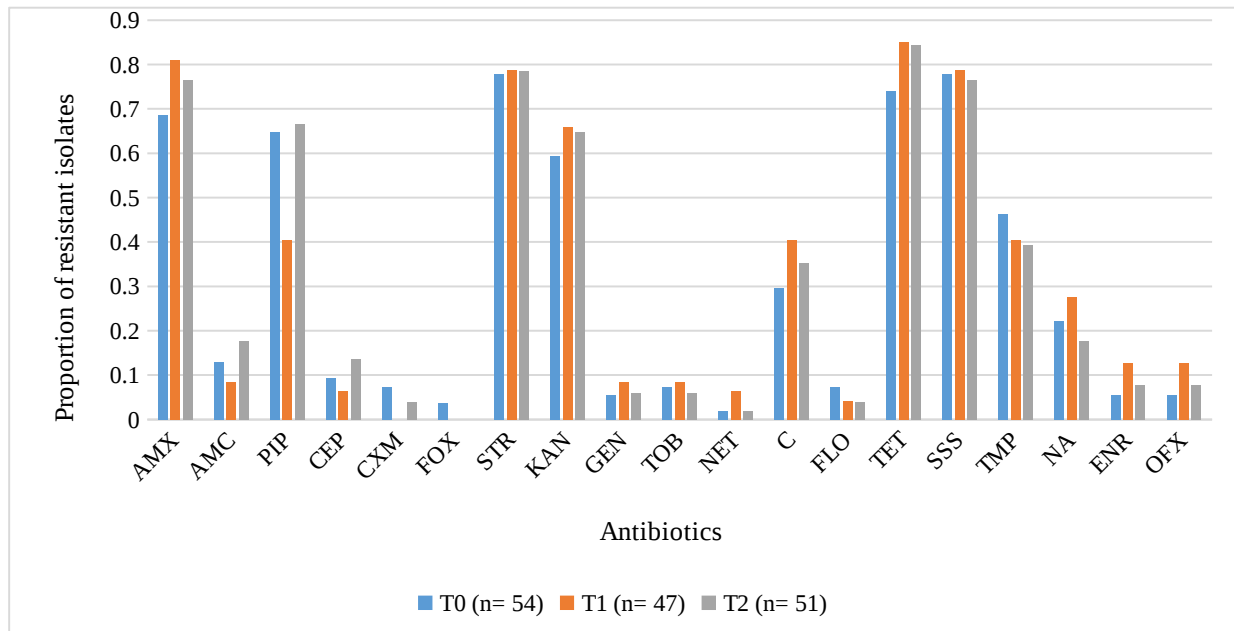


Figure S2. Proportion of resistant *E. coli* isolates before amoxicillin treatment (T0), at amoxicillin withdrawal (T1), one week after amoxicillin withdrawal (T2).

4. Analysis of calves' intestinal microbiota before and after amoxicillin treatment by shotgun sequencing

Stools from calves were collected and conserved following the Standard Operating Procedures (SOP) IHMS_SOP 03 V2³²⁶. Within the first 24h from collection, stools were separated in two aliquots of 300mg each and frozen at -80°C. DNA was extracted from stools following the SOPs IHMS_SOP 06 V2³²⁷. Quantity and quality of extracted DNA was evaluated using NanoDrop and Qubit technology. Library preparation and shotgun sequencing were outsourced to GetT-Plage (Toulouse). Sequencing was performed using a NovaSeq6000 sequencer, generating 2x150 bp paired-end reads. Generated reads ranged between 77 to 107 million in stools collected before amoxicillin treatment (T0); 68 to 106 million at the end of amoxicillin therapy (T1); and 45 to 87 million reads one week after T1 (T2).

Analysis of the data was realized using a pipeline developed for this study. The generated reads were cleaned from adaptators sequences used for the libraries construction using Cutadapt, followed by Sickle for trimming³²⁸ and Samtools for removing reads from host genome. Quality sequencing was also determined by the FastQC software before the assembly step. The assembly was obtained using MetaSPAdes³²⁹ and annotation using PROKKA software³³⁰. Annotated proteins were clustered in Operational Taxonomic Units (OTUs) using the cd-hit software³³¹. Taxonomic and abundance of the species were determined using Kaiju³³². Statistical analysis was conducted for α -diversity (Shannon index), for bacterial richness (S index) and β -diversity (Unifrac and Bray-Curtis tests) evaluation using R. The ARGs content was analyzed using the CARD database. SNPs (Single nucleotide polymorphism) conferring resistance to antibiotics (e.g. *gyrA* or *parC*) were also investigated³³³.

Preliminary analysis suggests that the number of OTUs observed varied between 600 and more than 800 at T0; 550 to 950 at T1 and 775 to 1300 at T2 (Figure 1). At T0, the predominant phylum was Firmicutes (50% of relative abundance), followed by Bacteroidetes, Actinobacteria and Proteobacteria. At T1, Firmicutes and Verrucomicrobia abundance decreased whereas Bacteroidetes, Actinobacteria and Proteobacteria abundance increased. One week later, at T2, Actinobacteria decreased and a slight increase of Firmicutes occurred. Verrucomicrobia did not return to the baseline (Figure 2). Data from control group not receiving amoxicillin are not yet available. The comparison to these data is crucial to interpret the effect of amoxicillin on IM composition. In fact, at the age of the calves enrolled in the study the IM composition is rapidly varying. However, these preliminary results are

comparable to findings obtained from piglets receiving amoxicillin by intramuscular injection^{334,335}.

Alpha diversity analysis showed non-significant changes in the Chao1, Shannon and InvSimpson index between the three samplings.

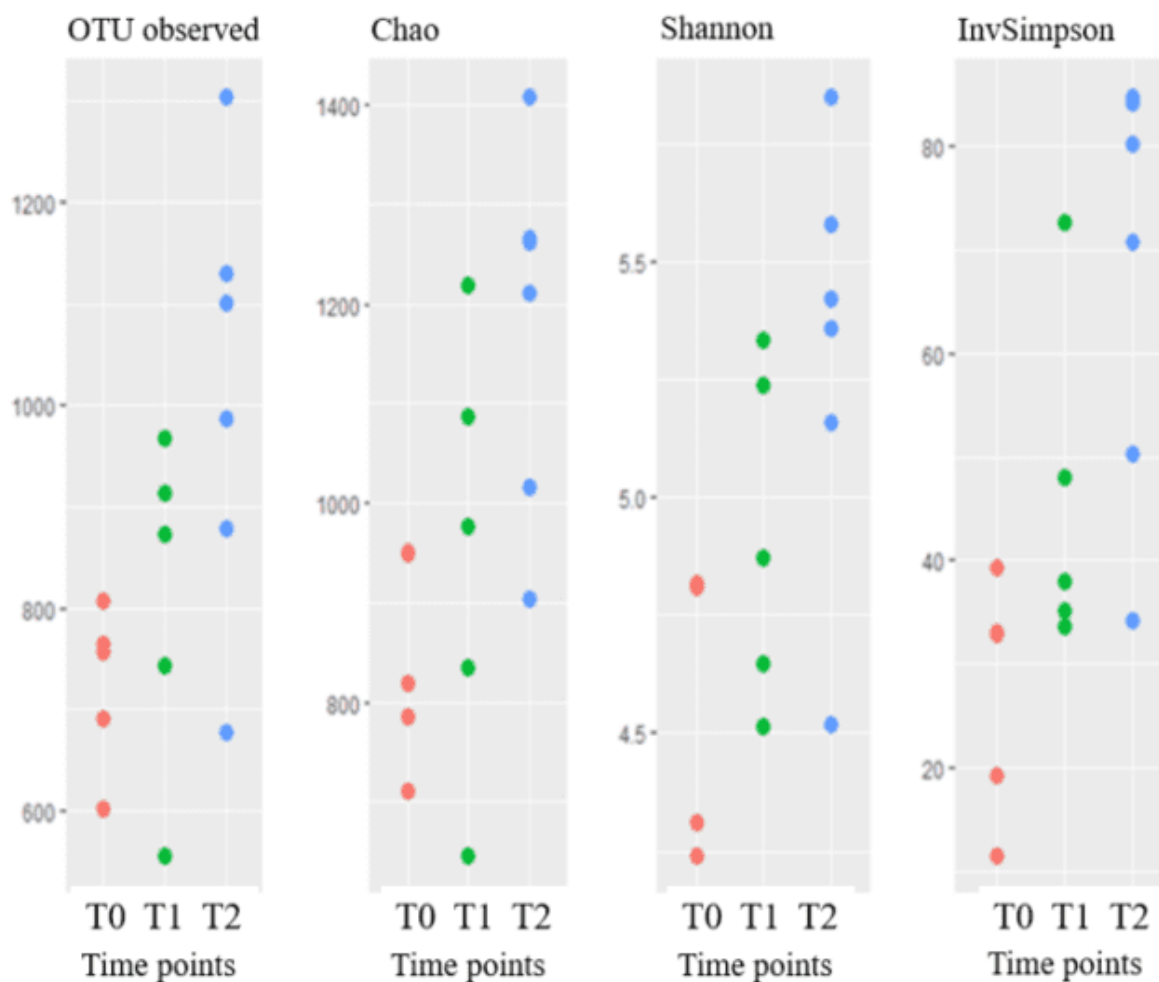


Figure 1: The difference in bacterial diversity indices (OTU observed, Chao 1, Shannon, and InvSimpson) measures between the treated calves at the three sampling times (T0; T1; T2).

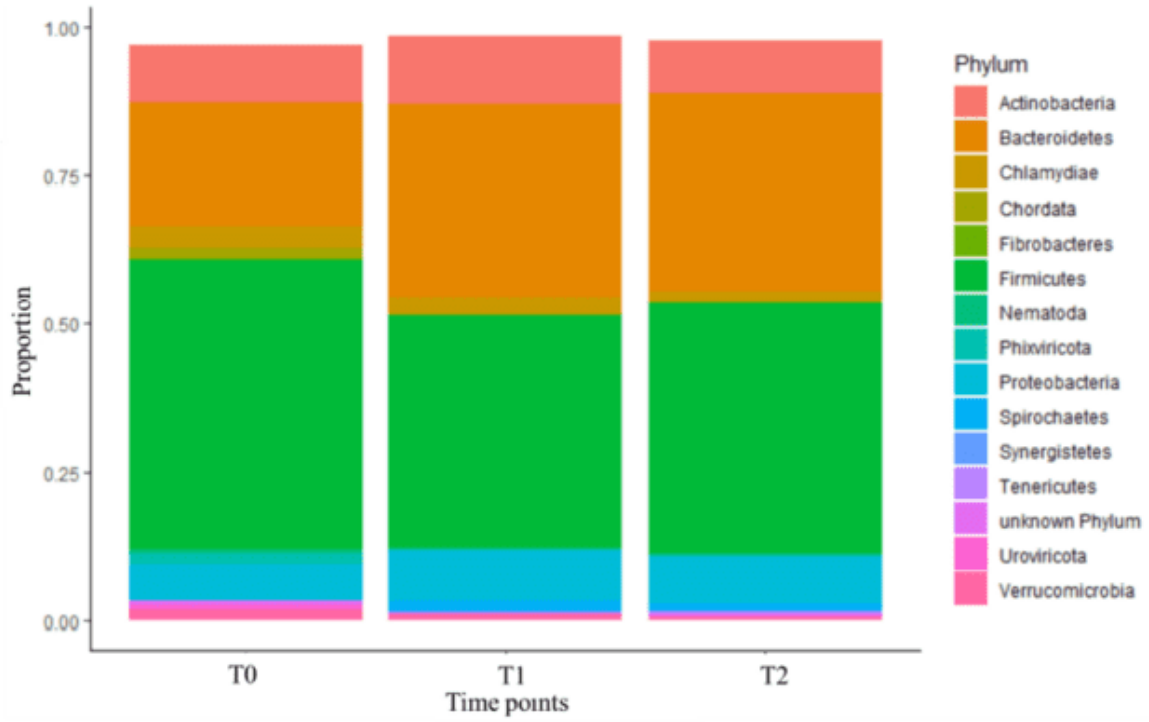


Figure 2: Taxonomic classification at the phylum level of IM of calves at T0 (before amoxicillin therapy); T1 (at amoxicillin therapy withdrawal), and T2 (one week after T1).



IV. General discussion

Bovines are major food-producing animals in Europe. In 2019, the European bovine livestock population consisted of 77 million animals mainly located in France (20 million), followed by Germany, the United Kingdom, Ireland, Spain, Poland and Italy³³⁶.

In France, diarrhea and respiratory tract infections are the most common infections occurring in calves requiring antibiotic treatment¹⁸. In 2019, the most sold antibiotics for the bovine sector were penicillins, followed by tetracyclines, aminoglycosides and macrolides. Overall, antibiotics sales for the bovine sector have decreased since 2011. In particular, after a decree established in 2014 about the restriction of antibiotic classes considered critical for human medicine, sales of 3rd and 4th generation cephalosporins and fluoroquinolones dropped a couple of years later³³⁷. In the same year, an increase in penicillins sales was observed, probably because of a reorientation of therapies based on 3rd and 4th generation cephalosporins towards older molecules. Restriction of usage for animals' therapies of critical antibiotics was a reasonable action for limiting the dissemination of ESBL-producing isolates that could evolve in animals and transfer to humans by direct contact or by exchange of genetic material supporting ESBL-encoding genes. This strategy, indeed, has contributed to a drastic reduction of ESBL-producing *E. coli* in bovines, in France¹⁸. Besides, the usage of old antibiotics could cause the allopathic selection of mutations causing the evolution of the spectrum of activity of a resistance mechanism towards newer molecules of the same class. The *bla*_{TEM-1} gene confers resistance to penicillins, but its variant *bla*_{TEM-52}, deriving from *bla*_{TEM-1} by three aminoacids substitutions Glu104Lys, Met182Thr, and Gly238Ser has acquired ESBL activity, thus towards 3rd and 4th generation cephalosporins. Under penicillins' selective pressure, acquisition of mutations leading to an ESBL phenotype of *bla*_{TEM} could be favored. Nowadays, the most common ESBL-encoding genes are of the *bla*_{CTX-M} type, but considering the almost ubiquitous dissemination of *bla*_{TEM-1}, the epidemiological situation of ESBL encoding genes could be reversed³³⁸.

In our study, the *bla*_{TEM} gene was present in all the stools of the calves studied. To analyze the effect of amoxicillin treatment on the IM composition and ARGs, stools from calves (n = 12) suffering from omphalitis and treated by intra-muscular injection of amoxicillin were collected before amoxicillin treatment, at the withdrawal of amoxicillin therapy, and one week after the withdrawal of the amoxicillin therapy. At the same pace, stools were collected from untreated calves (n = 5). Calves were aged between five and 26 days and were distributed in seven commercial farms. Quantification of ARGs, by qPCR using assays developed and validated in our laboratory, demonstrated that the *bla*_{TEM} number of copies/g feces increased in the feces of treated calves at the end of the treatment. In this

group, the amount of *bla*_{TEM} almost doubled at the end of the treatment, compared to the pretreatment sampling and it was eight folds higher than what was observed in the untreated group at the same sampling time. It seems that amoxicillin treatment, even by intra-muscular administration was able to reach the intestine and probably cause the selection of organisms harboring the *bla*_{TEM} gene. Amoxicillin is excreted unmodified in the urines at 80%, although a partial metabolization in the liver and excretion in the intestine by bile acid salts likely occurs. Selection of *bla*_{TEM} by intra-muscular administration of ampicillin, a molecule highly similar to amoxicillin, has been previously reported for pigs³³⁹. In our study, differences between the treated and the control untreated group did not show statistical significance. Comparison of *bla*_{TEM} amount at the pretreatment and the end of the treatment in the treated group was not significant, as well. Calves received different treatment regimens with variable duration and had age differences, a major driver of ARGs and IM composition in young animals. Also, the presence of *bla*_{TEM} in the control untreated calves and uncontrolled environmental conditions could have confounded amoxicillin effect. Besides, an increase of *bla*_{TEM} still occurred in treated calves. To evaluate if the amplification of the *bla*_{TEM} gene persisted after amoxicillin treatment, stools were collected and analyzed one week after the end of the treatment. At this sampling time, the level of *bla*_{TEM} decreased, even at lower levels than the one observed in the pretreatment sample. This observation suggests that the intestinal microbiota was recovering from the amoxicillin perturbation, at least concerning the amount of *bla*_{TEM}. While *bla*_{TEM} expanded in treated calves, depletion of the general bacterial population was observed. The preliminary metagenomics analysis by shotgun sequencing on the stools from six treated calves, suggests that, after amoxicillin treatment, the abundance of Firmicutes and Verrucomicrobia phyla decreased along with an increase of Bacteroidetes, Actinobacteria and Proteobacteria. This result is in agreement with several other studies, although performed on other animals or antibiotic treatments^{322,340–347}. One week after the amoxicillin withdrawal, the amount of Firmicutes raised again, whereas Verrucomicrobia remained less abundant than the pretreatment situation. In this phylum, microorganisms able to degrade mucin and fiber are included. This bacterial function is crucial for ruminants' health. Probably, the IM composition of treated calves might never return to the pretreatment situation not only because of perturbations caused by the amoxicillin treatment but also because in young animals, included calves, IM composition is extremely dynamic in the first weeks of life. Analysis of data from untreated calves will be crucial to understand the role of aging or amoxicillin in shaping the IM. Further analysis after T2 could have unveiled the eventual long-lasting consequences on the IM of calves and relation to their health status. In non-treated calves, the amount of the general bacterial population remained constant during

the sampling period according to the quantification of the 16S rRNA encoding gene, whereas the amount of *bla*_{TEM} decreased during the observation time. Previous studies have reported the decrease of ARGs in maturing IM, often associated with the decrease of the Proteobacteria phylum, in which ARGs of clinical importance are often found^{183,362}. During the sampling period, a decrease of viable, cultivable, Gram-negative bacteria was observed in most calves, including those not receiving the amoxicillin therapy, according to the enumeration of CFU on MacConkey agar. The decrease of CFU/g of feces observed by cultivation was not highlighted by the quantification of the 16S rDNA, which during sampling remained constant in calves not receiving amoxicillin. This discrepancy could be due to two principal factors. The cultivation condition selected for Gram-negative, aerobic, and of course viable bacteria. Identification of the obtained colonies pointed out that all belonged to the Enterobacterales family and, with few exceptions, to *E. coli*. The amplification of the 16S rDNA allowed the amplification of other bacterial families that could not be cultivated because of the chosen conditions. Furthermore, the qPCR could have amplified also the DNA of non-viable bacteria, does not evidencing the decrease evidenced by with cultivation. In treated calves, the amount of viable Gram-negative bacteria resistant or susceptible to amoxicillin was similar. This was the case for most untreated calves as well. Occurrence of amoxicillin resistance in *E. coli* from calves is known to be elevated¹⁸, and the occurrence of amoxicillin-resistant *E. coli* in the calves of this study is coherent with the French epidemiological situation.


The third axe of the project was conducted to enhance the sensitivity of the analysis compared to the shotgun sequencing and for giving information on the viable part of selected bacteria. Overall, 152 *E. coli* isolates were analyzed by next-generation sequencing and a comparable number of isolates was obtained at the three samplings. Most isolates (75%) were amoxicillin resistant. The *bla*_{TEM} gene was found in all amoxicillin-resistant isolates, whereas no *bla*_{SHV}, the second most common gene conferring amoxicillin resistance, was found in agreement with qPCR results. The *bla*_{TEM} gene was always located downstream of an insertion sequence encoding gene of the IS26 or IS91 type or close to Tn3 transposon encoding genes. All these genetic elements have been previously associated with ARGs. IS26 is a crucial genetic element for mobilization of ARGs³⁴⁸, besides mediating the evolution of other genetic elements, such as plasmids, possessing ARGs³⁴⁹. Tn3 transposon has large host spectrum that has been implicated in the mobilization of the *bla*_{TEM-1} gene and other ARGs in numerous bacterial species^{350,351}. In some isolates, *bla*_{TEM-1} was found close or into a *mer* operon, which encodes structures for cell detoxification from mercury ions. The presence of this operon in

bacteria colonizing food-producing animals could favor their survival in the intestine of the animal even in presence of mercury-containing pesticides or herbicides that could be ingested through contaminated food. Elements conferring resistance to heavy metals have been reported in *E. coli* of other sources including poultry, but also humans³⁵², and also from other several bacterial species³⁵³. These detoxification systems are ancient and necessary to the physiology of the cell. Their presence seems favoring the co-selection of ARGs in environmental compartments³⁵⁴.

In the isolates of this study, *intI1* gene was detected frequently and always associated with a resistance cassette, with *dfrA5* as the most common. A correlation between class 1 integrons and the occurrence of *tet* genes, in particular *tetA* and *tetB* genes, has been previously highlighted³⁵⁵. Isolates of our study possessing class 1 integrons carried *tetA* genes, as well. According to a bioinformatic prediction, all the ARGs found in this study were located mainly on plasmids. This is in agreement with a previous observation that described *bla*_{TEM} mostly located on IncF type plasmids^{322,323}. However, further analysis is ongoing to confirm this plasmid localization and characterization of plasmid types.

In several calves, isolates surviving amoxicillin therapy and differing for a few SNPs (< 10) were found. Interpretation of clonality based on SNPs occurrence is not formalized, it depends on the epidemiological situation and sampling sources. No more than 10 SNPs have been reported in isolates collected consecutively from the same host during 12 months. Based on this observation, it could be assumed that isolates from the same calf, found at different sampling times and presenting less than 10 SNPs, most likely belong to the same clone. According to the current analysis, these clones did not differ for the content of adhesins or other virulence factors that could have favored the persistence in the calf, besides, of course, the presence of *bla*_{TEM}. These clones belonged to ST58, 362, 949, 117, 69, 48 and 23, which are global spread sequence types. The duration of colonization of humans with multidrug-resistant clones is not long-lasting (three months)³⁵⁶. In our study, two calves that lived on the same farm, but not contemporaneously (at two years of distance), were found colonized by the same clone. This phenomenon was observed in farm A. Persisting contamination of the farm environment cannot be excluded. Cellular features that could favor this persistence could be the ability of the clone to resist to desiccation or biofilm formation. Persistence of ESBL-producing *E. coli* has been previously reported³⁵⁷. However, the farmer or other animals living in the farm and colonized by the clone could be vectors and reservoirs for propagation to further hosts. A deeper analysis of these clones comparing their similarity with sequences from genomic databases could be useful to understand their origin, for instance to unveil if

similar clones were found in farmers or more commonly in environmental sources. We do not know if the calves were born from the same mother, which could be the epidemiological link. In the recent past, much attention has been focused in the role of animals as a source of multidrug-resistant bacteria for humans and indeed resistance mechanisms evolved in food-producing animals have been transmitted to farmers^{358,359}. However, the contamination of animals from humans could occur, as well, thus hygienic measures should prevent bacteria shuffling in both directions. Genomically similar isolates were found in calves residing in different farms. The number of SNPs differentiating these isolates decreased with the decrease of the geographical distance between farms. This suggests that a common reservoir of colonization for calves is likely to exist. Candidate to this role could be several elements. Contaminated groundwater used for calves watering or food, for instance birds, wild animals and insects³⁶⁰ could be living vectors that could disseminate clones among relatively distant farms³⁶¹. Role of flies in the dissemination of AMR is increasingly recognized. Limiting the exposure of feces to open-air could limit bacterial carriage by flies and thus dissemination of resistances.



V. Conclusion and perspectives

Integrating multiple methodological axes, our study put the base to understand the role of amoxicillin therapy on the IM and resistances of calves. The impact of antibiotic therapy needs to be known to mitigate negative consequences on the host health and to counteract the selection of ARGs in the intestine. From an extensive bibliography search, negative effects of necessary antibiotic therapies could be mitigated by the administration route, with parenteral administration showing less effect than orally administered antibiotics. In France, most of antibiotics are administered by parenteral route in bovines, except for tetracycline that is orally administered.

Further studies are needed on the pharmacology and pharmacokinetics of the current available antibiotics in animals to understand how parenterally administered antibiotics reach the intestine and in which amount. Furthermore, they could help improving therapeutical dosage and regimens, duration of therapy, harmonizing prescription practices in veterinary medicine.

Studies analyzing the effects of antibiotics on the IM and ARGs should be conducted in both environmental-controlled farms and commercial farms. The first set-up could attribute variation on the IM and ARGs to the antibiotic more straightforwardly, as confounding factors like age of the host, nutrition, antibiotic treatment in other hosts living in the same farm, contamination of drinking water or food by antibiotics could be better managed.

Studies in commercial farms are necessary, as well. In our study, calves enrolled in the project did not harbor bacteria with major resistance mechanisms, in terms of relevance for human clinics, except for few isolates that demonstrated fluoroquinolones resistance. However, several isolates demonstrated resistance to multiple classes of antibiotics, thus defined as multidrug-resistant. In the veterinary clinic, these resistances could limit the therapeutical options for treatment. Some clones were able to survive to amoxicillin treatment and to disseminate to other calves. To understand the dynamic of clones dissemination and persistence in the farm environment and interaction with antibiotic selective pressure, field studies need to be incremented. In this context, a holistic approach should be implemented analyzing animals, environmental compartments of the farm, including bedding, soil, feeding dispositive, water and the possible reservoir of AMR living in the farm (farmers, domestic animals, flies). Furthermore, residues of antibiotics should be estimated in other sources than the therapy such as water and food.

Investigation on co-selection of AMR are needed. Role of heavy metals has been neglected in the farm environment, however, they could reach food-producing animals

conjugated to pesticides and herbicides, through water, food and grassland. Next-generation sequencing technologies will facilitate unveiling the genetic basis of this ancient bacterial function in connection with antibiotic resistance.

In this study, qPCR produced informative results and did not require advanced bioinformatics skills and support. On the light of results obtained on the cultivation of bacteria, the current system could be amended by adding opportune targets for the quantification of *E. coli*. However, studies on the IM should be preferentially based on shotgun sequencing methodology. Considering the similarity of IM among mammals, the proportion of occurring phyla should be clearly reported to appreciate differences among different hosts and in longitudinal studies. Obtaining the complete picture not only of the IM composition and its resistances but also of bacterial functions, could open to IM modulation strategies to mitigate antibiotics' negative effects.



VI. References

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