Master's Degree in Animal Genetics and Breeding, and Reproductive Biotechnology

DAIRY CATTLE RUMINAL RESISTOME: CHARACTERISATION AND ASSOCIATION WITH PRODUCTIVE TRAITS

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1. Communication to conferences

The results obtained in this thesis have been accepted and will be presented in the 71th EAAP Annual Meeting 2020, Porto, Portugal.

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This work has also been awarded with an EAAP 2020 Scholarship.

The results of this thesis will also be presented in the World Buiatrics Conference in Madrid in 2021.

2. Abstract

The rumen resistome is the compound of all the antimicrobial resistance genes (ARGs) present in the microbes that inhabit the rumen. The World Health Organization (WHO) warned about the issue with antimicrobial resistant (AMRs) pathogens, as it is predicted that by 2050 multi-resistant bacteria will kill 10 million people per year, surpassing cancer as our main health concern.

Among the 1,461 diseases recognised in humans, 60% of them are caused by multi- host pathogens capable of moving across species. And roughly 75% of the newly detected infective diseases over the last 30 years have been zoonotic. Here lies the importance of characterizing the rumen resistome, as ARGs could jump from faeces and saliva within and across species, arriving to humans via direct contact, through the food chain or disseminated in the environment (e.g. manure). A good approach to reduce the risks of the emergence of AMR is to understand how they appear, their relationship with the host, how they interact or how they are transmitted to humans. This is one of the goals of the One Health Initiative, which is the integration of human, animal and environmental health under the same framework.

This thesis aims to contribute to this Initiative by characterizing the rumen resistome and estimating the host genetic control over thereof. The ruminal metagenome of 472 Friesian cows from 14 commercial Spanish farms were sequenced using the MinION device from Oxford Nanopore Technologies. After quality control, the DNA reads were analysed with the SQMreads tool from SqueezeMeta (Tamames and Puente-Sánchez, 2019), a pipeline for metagenomics. Aligning each read to a gene reference database and providing the number of copies of each gene present in the sample. We implemented a custom integration of the Comprehensive Antibiotic Resistance Database (CARD). The pipeline was implemented in the CESGA super-computing centre in Galicia.

As a result, the 69 most prevalent ARGs were determined. The heritability of the relative abundance of the more abundant ARGs ranged between 0.10 (*mupA*) and 0.49 (*tetW*). The most remarkable correlations were found between *msbA* and methane emissions(- 0.45), *rpoB2* and fat yield (-0.62) and *macB* and methane emission (-0.40).

Twenty-five of these genes were analysed individually and their phenotypic correlation with bacteriophages was calculated, showing that their relative abundances are highly correlated between them and with the bacteriophages. This can be explained by the presence of a multidrug resistant plasmid and a high horizontal gene transfer mediated by bacteriophages.

As a conclusion, we were able to determine the most prevalent ARGs in the ruminal ecosystem, and their relative abundance in the rumen resulted to be highly heritable, with strong genetic correlation with economically important traits. Further studies are needed to gain insights on the role of these genes in the rumen metagenome.

3. Resumen

El resistoma ruminal está compuesto por todos los genes de resistencia antimicrobiana (ARG) presentes en los microorganismos que habitan en el rumen. La Organización Mundial de la Salud advirtió sobre el problema de los patógenos resistentes a antimicrobianos (AMR), ya que se prevé que para 2050 las bacterias multirresistentes dejarán 10 millones de víctimas al año, superando al cáncer como nuestro principal problema de salud.

Entre las 1,461 enfermedades reconocidas en humanos, el 60% de ellas son causadas por patógenos de múltiples huéspedes capaces de moverse a través de especies. Aproximadamente el 75% de las enfermedades infecciosas recientemente detectadas han sido zoonóticas. Aquí radica la importancia de caracterizar el resistoma ruminal, ya que los ARG podrían saltar de las heces y la saliva tanto inter como intra-especies, llegando a los humanos por contacto directo, a través de la cadena alimentaria o diseminados en el medio ambiente (por ejemplo, estiércol). Un buen enfoque para reducir los riesgos de la aparición de AMR es comprender cómo aparecen, su relación con el huésped, cómo interactúan o cómo se transmiten a los humanos. Este es uno de los objetivos de la Iniciativa Una Única Salud (One Health Initiative) que busca la integración de la salud humana, animal y ambiental bajo el mismo marco.

Esta tesis tiene como objetivo caracterizar el resistoma ruminal y determinar la la heredabilidad de la composición de este resistoma en el vacuno lechero. Para ello, el metagenoma ruminal de 472 vacas frisonas de 14 granjas comerciales españolas fue secuenciado usando el dispositivo MinION de Oxford Nanopore Technologies. Después del control de calidad, las lecturas de ADN se analizaron con la herramienta SQMreads de SqueezeMeta (Tamames y Puente-Sánchez, 2019), una pipeline para metagenómica, que alinea cada lectura con una base de datos de referencia de genes y proporcionando el número de copias de cada gen presente en la muestra. Implementamos una integración personalizada de la base de datos integral de resistencia a antibióticos (CARD). El pipeline se implementó en el centro de supercomputación CESGA.

Como resultado, se determinaron los 69 ARG más prevalentes. La heredabilidad de la abundancia relativa de los ARG más abundantes osciló entre 0,10 (mupA) y 0,49 (tetW). Las correlaciones más notables se encontraron entre msbA y metano (- 0.45), rpoB2 y rendimiento de grasa (-0.62) y macB y emisión de metano (-0.40).

Se analizaron 25 de estos genes individualmente y se calculó la correlación fenotípica entre su abundancia fenotípica y las de los bacteriófagos, observando altas correlaciones. Esto puede explicarse por la presencia de plásmidos resistente a múltiples fármacos y a una alta transferencia horizontal de genes mediada por bacteriófagos.

Como conclusión, pudimos determinar los ARG más prevalentes en el ecosistema ruminal, que mostraron una alta heredabilidad de su abundancia relativa, con una fuerte correlación genética con carácteres productivos de importancia económica. Se necesitan más estudios para obtener información sobre el papel de estos genes en el metagenoma ruminal.

4. Resum

El resistoma ruminal està compost per tots els gens de resistència antimicrobiana (ARG) presents en els microorganismes que habiten en el rumen. L'Organització Mundial de la Salut va advertir sobre el problema dels patògens resistents a antimicrobians (AMR), ja que es preveu que per a 2050 els bacteris multirresistentes deixaran 10 milions de matesal any, superant al càncer com el nostre principal problema de salut.
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El metagenoma ruminal de 472 vaques frisones de 14 granges comercials espanyoles va ser seqüenciat usant el dispositiu miniàs d'Oxford Nanopore Technologies. Després del control de qualitat, les lectures d'ADN es van analitzar amb la ferramenta SQMreads de SqueezeMeta (Tamames i Puente-Sánchez, 2019), una pipeline per a metagenómica. Alineant cada lectura amb una base de dades de referència de gens i proporcionant el nombre de còpies de cada gen present en la mostra. Implementem una integració personalitzada de la Base de dades integral de resistència a antibiòtics (CARD). La pipeline es va implementar en el centre de supercomputació CESGA. Com resultat, es van determinar els 69 ARG més prevalents. L'heredabilidad de l'abundància relativa dels ARG més abundants va oscil·lar entre 0,10 (mupA) i 0,49 (tetW) . Les correlacions més notables es van trobar entre msbA i metà (- 0.45) , rpoB2 i rendiment de greix (-0.62) i macB i emissió de metà (-0.40).
Es van analitzar 25 d'estos gens individualment i es va calcular la seua correlació fenotípica amb bacteriòfags, la qual cosa va demostrar que estan altament correlacionats entre ells i amb els propis bacteriòfags. Açò pot explicar-se per la presència d'un plasmidi resistent a múltiples fàrmacs i a una alta transferència horitzontal de gens mediada per bacteriòfags. Com a conclusió, vam poder determinar els ARG més prevalents en l'ecosistema ruminal, que van mostrar una alta heredabilidad de la seua abundància relativa, amb una forta correlació genètica amb trets econòmicament importants. Es necessiten més estudis per a obtindre informació sobre el paper d'estos gens en el metagenoma ruminal.

5. Index

6. What is antimicrobial resistance?

The most remarkable role of modern medicine is being able to prevent and cure life-threatening diseases and infections, which is becoming a problem as antimicrobial-resistant (AMRs) pathogens are gaining prevalence. The World Health Organisation (WHO), the World Organisation for Animal Health (OIE) and the Food and Agriculture Organisation, all together known as the Tripartite Collaboration, proposed an action plan with five strategies to approach this issue: (1) to improve awareness and understanding of antimicrobial resistance; (2) to strengthen knowledge through surveillance and research; (3) to reduce the incidence of infection; (4) to optimize the use of antimicrobial agents; and (5) to ensure sustainable investment in countering antimicrobial resistance (WHO, 2017, 2019). They asked all countries for creating and implementing multisectoral national action plans to address the AMR problem, as the main priority may differ between countries. For instance, getting rid of the disease burdens, improving the human-animal interactions and environmental practises such as wastewater disposal and sanitation.

The WHO warned about the imminent problem of AMRs. It is predicted that by 2050, AMRs will become the first cause of death globally, killing more than 10 million people per year and surpassing cancer as our main health concern. Tackling AMRs before the microbial passes to humans will help improving global health by avoiding new infections resistant to antimicrobials.

Among the 1,461 diseases recognised in humans, 60% of them are caused by multi-host pathogens capable of moving across species. Roughly 75% of the newly detected infective diseases over the last 30 years have been zoonotic. (Taylor et al., 2001).

Most of the current antimicrobial compounds are inhibitory to Gram-positive bacteria such as *Eubacterium, Lactobacillus, Butyrivibrio, Lachnospira* and *Ruminococcus*. The main issue is rising among Gram-negative bacteria like *Bacteroidetes, Megasphaera, Selenomonas, Succinimonas, Succinivibrio* and *Veillonella*. These bacteria are gaining antimicrobial resistance genes (ARGs), becoming resistant or multi-resistant to diverse drugs such as avoparcin, narasin, salinomycin, thiopeptin, tylosin, virginiamycin, and ionophore antibiotics like RO22-6924/004 and RO21-6447/009 (Nagaraja and Taylor, 1987). The bacteria listed above are mutualists or commensalistic, so the risk of them acquiring resistance to antimicrobials comes from using antibiotics (ATBs) as growth promoters. In dairy cattle, the use of ATB with prophylactic aims is forbidden as milk could contain ATBs residues. In this case, the problem lays within the use of ATB to treat diseases such as mastitis, lameness, respiratory diseases or metritis.

Using antimicrobial feed additives on livestock to promote the growth was prohibited in Europe in 2006, but it is still a problem in other countries as it increases the abundance of antimicrobial resistance genes (ARG) in the gut and faeces (Penders et al., 2013; Reddy et al., 2014). This can become a problem if we think about the use of livestock faeces as manure and organic fertiliser. It is important to understand that bacteria have mobile genetic elements (MGE) such as transposons and plasmids as well as other ways as horizontal gene transfer (HGT) or bacteriophages to pass genetic material to other species. ARGs could

jump from the faeces to the soil bacteria, then to plants, and then to herbivores which products are eaten by humans (Zhang et al., 2013). This is turning into an important issue to address, as antibiotic-resistant microbes originated in farms are becoming a threat to human health as well as the resistances originated at human levels will be a threat for the animal and environmental health.

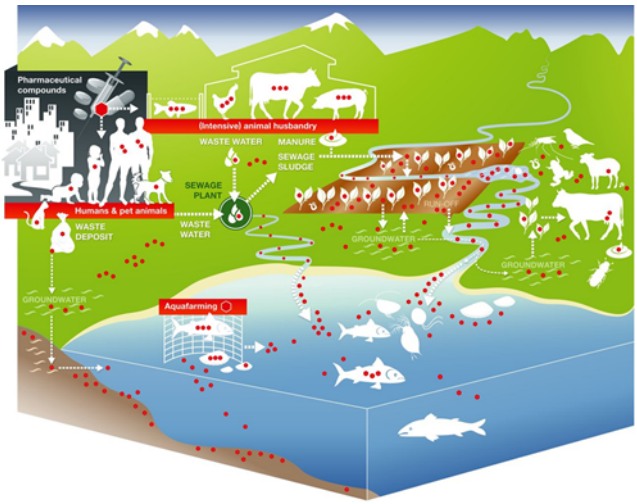

Figure 1 | **Spread pathways for AMR**. | Taken from (Berkner et al., 2014)

A good approach to reduce the risks of the emergence of AMR is to understand how they appear, their relationship with the host, how they interact amongst themselves and how they are transmitted to humans.
AMRs represent a problem to the livestock industry not only for the inherent risk that represents for both animal and human health but also because of the increase of morbidity and mortality coming together with the appearance of new microorganisms with ARGs. This is an added cost to the industry as new remedies should be developed (Mathew et al., 2007).
Regarding the health risk mentioned above, we must consider that there are two possible ways for AMR to infect humans. The first one will be direct exposure to antimicrobial-resistant bacteria by contacting with host animals (livestock and pets) (Price et al., 2007) and the second one via the food chain by contamination of the meat by resistant commensal and pathogenic AMRs (van den Bogaard and Stobberingh, 2000).
The use of antimicrobials as treatment, and as prevention and improvement of production rates outside the European Union can promote AMRs by two main reasons: the increase of the number of AMRs mediated by the selective pressure of those resistant strains and by the dissemination of the ARG horizontally carried by plasmids or transposons.
It is also interesting to study the quorum and fucose sensing (Hughes et al., 2010; Pickard et al., 2014) as they act as interkingdom signalling pathways (Curtis and Sperandio, 2011) to regulate bacterial colonisation and virulence within the host (Roehe et al., 2016a). If we understand these mechanisms, we could deceive these microorganisms to control or decrease their populations using faux signals.

6.1. One Health Initiative

The One Health concept aims to create interdisciplinary strategies to improve the healthcare of humans, animals and the environment. The initiative encourages physicians, osteopathic physicians, veterinarians, dentists, nurses and other scientific-health and environmentally related disciplines to share information and work together towards solutions for big health problems. This project is based on the premise that human health, animal health, and aquatic and terrestirial environment health are linked, making One Health Initiative (OHI) the best approach to improve the well-being of all species. There are seven statements to follow (OHI - One World One Medicine One Health):

1. Joint educational efforts between human medical, veterinary medical schools, and schools of public health and the environment;
2. Joint communication efforts in journals, at conferences, and via allied health networks;
3. Joint efforts in clinical care through the assessment, treatment and prevention of cross-species disease transmission;
4. Joint cross-species disease surveillance and control efforts in public health;
5. Joint efforts in better understanding of cross-species disease transmission through comparative medicine and environmental research;
6. Joint efforts in the development and evaluation of new diagnostic methods, medicines and vaccines for the prevention and control of diseases across species and;
7. Joint efforts to inform and educate political leaders and the public sector through accurate media publications.

Because of this matter, the European Food Safety Authority (EFSA) has proposed a plan to gain insight into microbiota and antimicrobial resistance. The 2020 action plan aims to integrate the new findings in gut microbiota and antimicrobial resistances to EFSA's scientific assessments (European Food Safety Authority, 2016). Since 2017, there is a coordinated plan on combating anti-microbial resistance with the European Commission, sister agencies and Member States. It was included under the Biological Risk Assessment the study of the microbiome, the antimicrobial resistances and animal-based indicators for animal welfare. This is a new field of study that opens plenty of new opportunities to approach issues that suppose a risk in near future.

We must find an intermediate position between completely removing antibiotics from animal production and the current state of the situation. If we slowly withdraw them from the industry, the transmission to humans could decrease (Stokes et al., 2008). Completely taking them off the farms would drive to a worsen of animal health and condition, and to an increase in the pathogen load, exhibiting a new threat to human health. It is necessary to limit its usage to well-diagnosed cases and to improve early detection of bacterial diseases (Cox and Popken, 2006).

7. Types of AMR and their underlying molecular mechanisms

7.1. Degradation by enzymes

This mechanism works by breaking down a relevant part of the antibiotic molecule to inactivate it *(Figure 2b)*. Some bacteria have the enzyme β-lactamase which degrades the β-lactam ring of antibiotics belonging to the β-lactam group such as penicillin derivates, cephalosporins, monobactams and carbapenems (Keith B. Holten, M.D, and Edward M. Onusko, 1970). Addition of acetyl-CoA by acetyltransferase also occurs to acetylate antibiotics which have hydroxyl groups in their chloramphenicol molecules. Furthermore, aminoglycosides can be inactivated by the addition of acetyl-CoA, adenylyl groups or phosphates by acetyl, adenyl and phosphotransferases (Trott, 2013; Rubin and Pitout, 2014).

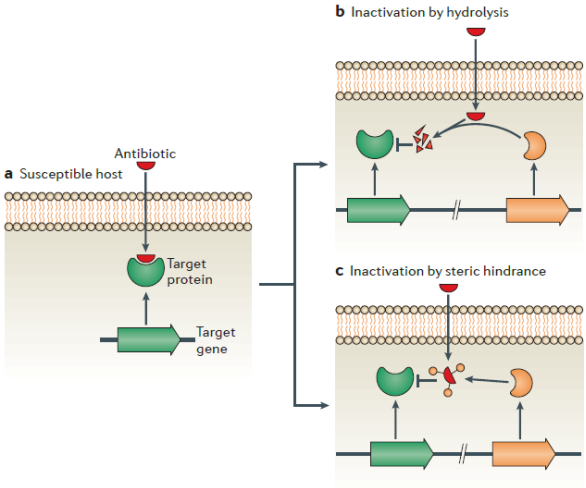

Figure 2 | **Direct interactions with antibiotics. a** | Susceptible host representation. The target is inhibited by the antimicrobial. **b** | Resistance obtained by degradation of the antimicrobial molecule by an enzyme. **c** | Modification of the antimicrobial molecule by enzymes, preventing the binding with its target. Taken from (Blair et al., 2015).

7.2. Target modification

Antimicrobial molecules bind to a given structure known as targets to interrupt a metabolic pathway. If the target conformation changes, the antimicrobial will not be able to bind, and thus, to stop the metabolic process *(Figure 3c)*. Usually, alterations in ribosomal binding sites reduce the activity of antibiotics (Spratt, 1994). Resistance to macrolides and phenolics is mainly determined by this phenomenon.

Target site changes can appear due to spontaneous mutations on the bacterial chromosome in presence of the antimicrobials. Mutations in RNA polymerase lead to resistance to rifamycin and in DNA gyrase to quinolones. It can also happen by HGT leading to the acquisition of *mecA* genes that confers resistance

to methicillin in *Staphylococcus aureus* and genes in enterococci conferring resistance to glycopeptides (Lambert, 2005).

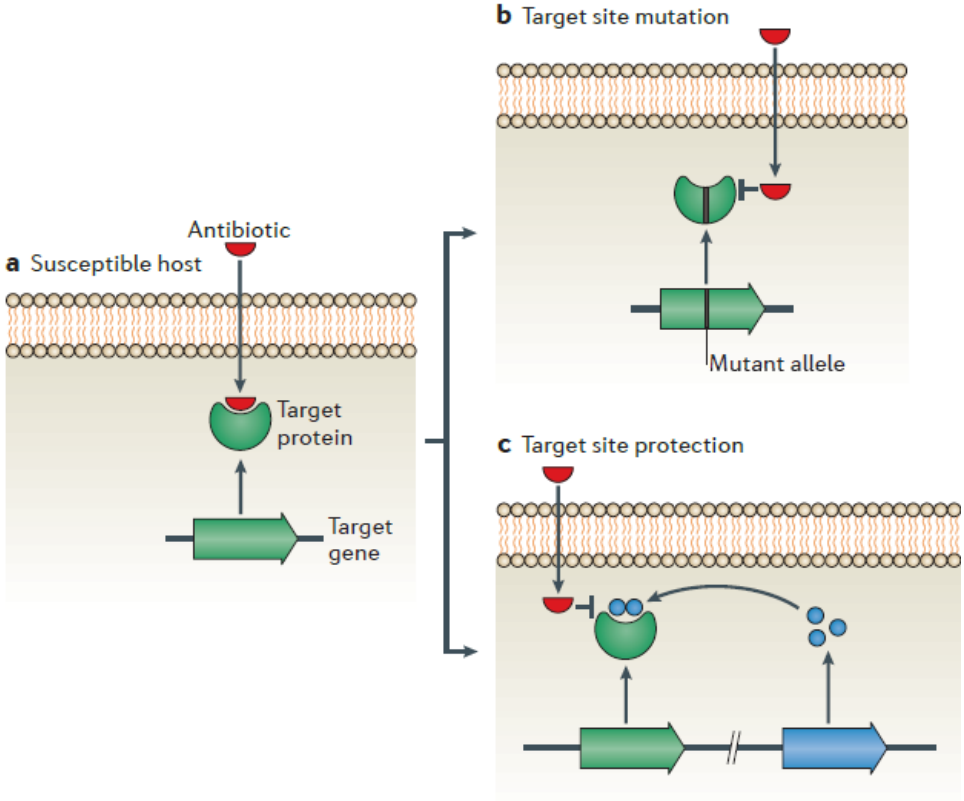

Figure 3 | **Target site changes. a** | The antibiotic can bind to its target and play its role as an inhibitor. **b** | Mutation of the target or recombination of alleles results in a reduced affinity for the antibiotic, which does not bind properly. The antibiotic effect is reduced or lost. **c** | The target can also be modified by the addition of a chemical group which will prevent the binding but retain its natural activity. Taken from (Blair et al., 2015).

7.3. Reduced permeability to drugs

The membrane permeability of Gram-negative bacteria adapts quickly under antibiotic stress and serves as a survival response. This is a very complex process regulated by pH, chemical stress and osmotic shocks.

A decrease in the membrane permeability reduces the accumulation of antimicrobials within bacteria, giving time to these microorganisms to develop other resistance mechanisms like target modification or drug inactivation *(Figure 4)*.

Porin size in the outer membrane affects to the permeability to antibiotics *(Figure 5)*. Gram-negative bacteria have extremely small-sized porins which hinder the pass of the molecules to the inside of the cell. Other bacteria like *Mycobacterium* have a thick layer of mycolic acid, a wax-like compound that obstructs the pass of the antibiotics (Pagès et al., 2008).

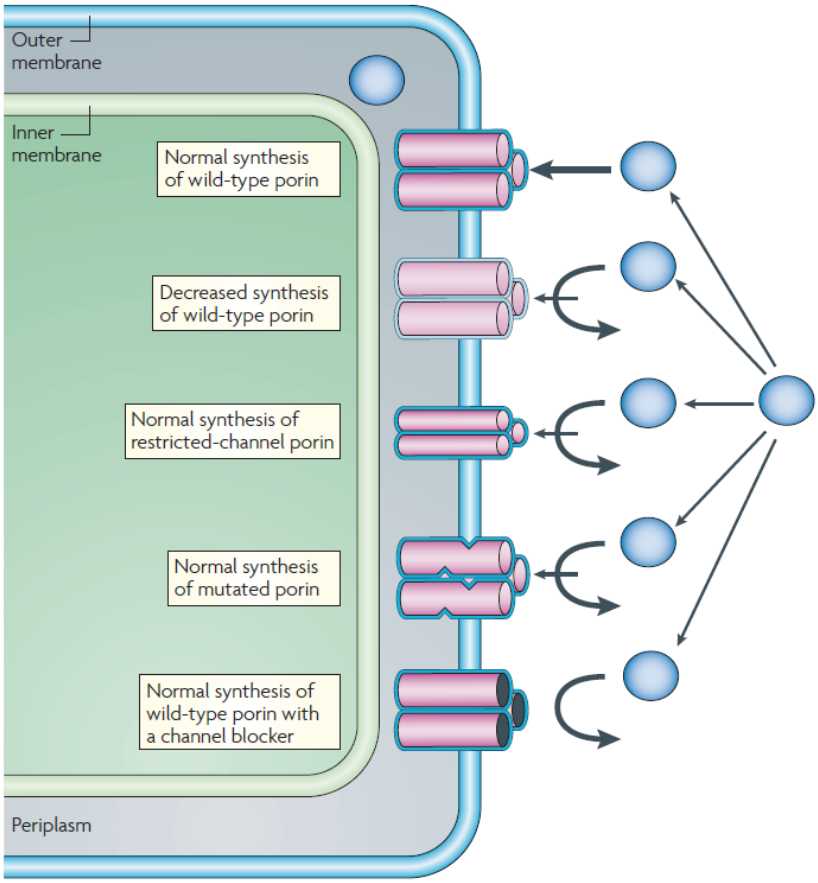

Figure 4 | **Resistance mechanisms associated with porin modification.**| Thickness of the arrows represents the level of the blue molecules through porin channels. Curved arrows show the uptake failure. Taken from (Pagès et al., 2008).

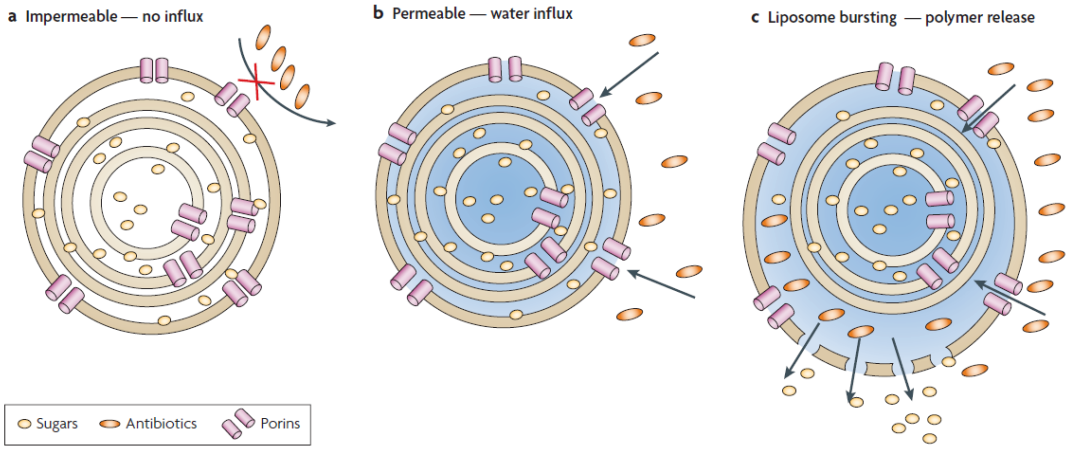

Figure 5 | **Permeability assay. a** | Liposomes are mixed in solution free of polymers and containing molecules of interests (antibiotics) with the same osmotic pressure. **b** | When the molecule permeates, it will create an osmotic gradient and water will swell the liposome. **c** | The liposome burst and release the polymer, leading to a decrease in optical density. This allows researchers to calculate the permeation rate. Taken from (Pagès et al., 2008).

7.4. Increase drug efflux

Resistance to tetracyclines, aminoglycosides and sulfonamides is often acquired by actively pumping the drugs out the bacterial walls *(figure 6)*. This reduces the concentration of the antimicrobial to levels where bacteria can grow and reproduce. *Pseudomonas spp., Staphylococcus aureus* and *Escherichia coli* are some examples of bacteria that use this mechanism (Brincat et al., 2011).
Staphylococcus aureus obtains its resistance to certain drugs thanks to the membrane protein NorA. This protein reduces the inner concentration of norfloxacin and other fluoroquinolones by actively transporting them out of the cell. Its activity depends on the transmembrane proton gradient (Neyfakh et al., 1993). NorA overexpression in *E. coli* has shown an increase in the drug efflux, leading to the belief that it is greatly involved in antimicrobial resistance itself or by association with other transporters (Yu et al., 2002).

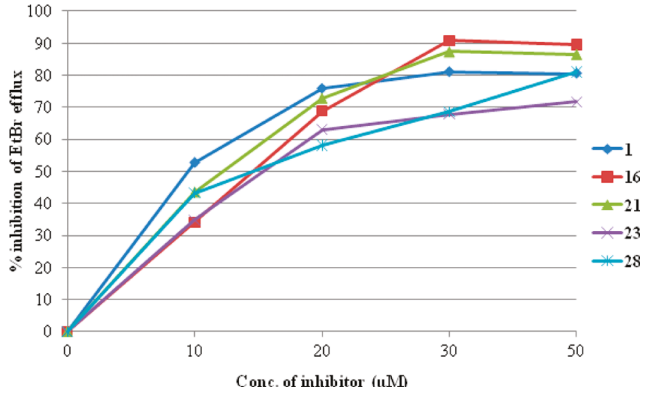

Figure 6 | **Dose-response curve of Ethidium Bromide efflux inhibition for the next figure compounds.** | Taken from (Brincat et al., 2011)

Quinolones are used as antibacterial compounds for pump-related resistant strains. These molecules inhibit NorA, restoring the efficacy of the compounds unless the bacterium presents other resistance mechanisms *(figure 7)*. Some examples of quinolones are 1,4-benzothiazine derivatives, N-piperine analogues, flavonolignan and flavone compounds, 2-aryl-5-nitro-1H-indoles, omeprazole analogues (Vidaillac et al., 2007), fluoroquinolone and 6-amino-8-methylquinolone ester derivatives (Samosorn et al., 2006).

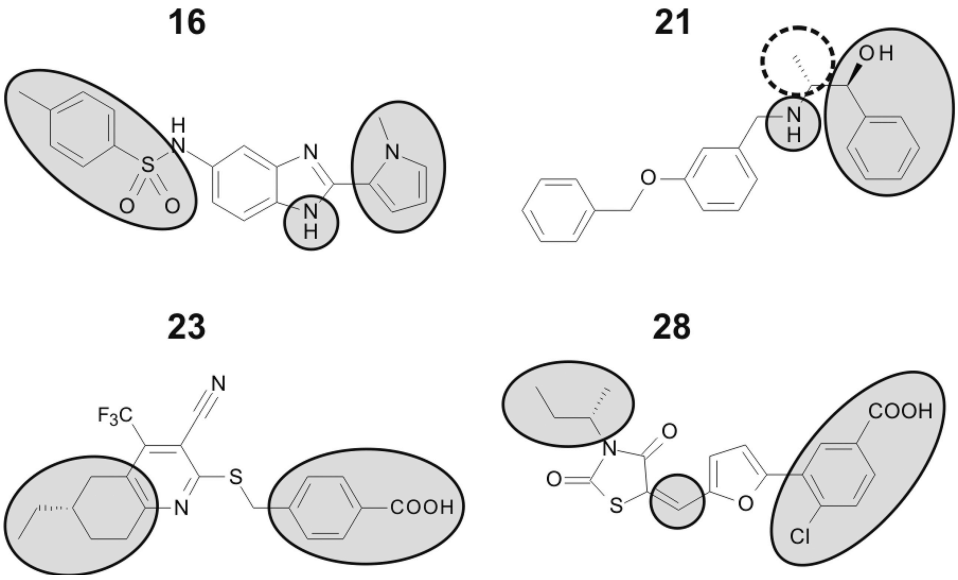

Figure 7. | **General structure of NorA inhibitory compounds.** | Taken from (Brincat et al., 2011)

Efflux pumps are classified into 6 different groups according to the number of transmembrane-spanning regions, source of energy used, number of components and types of molecules that the pump exports (*figure 8*) (Soto, 2013).

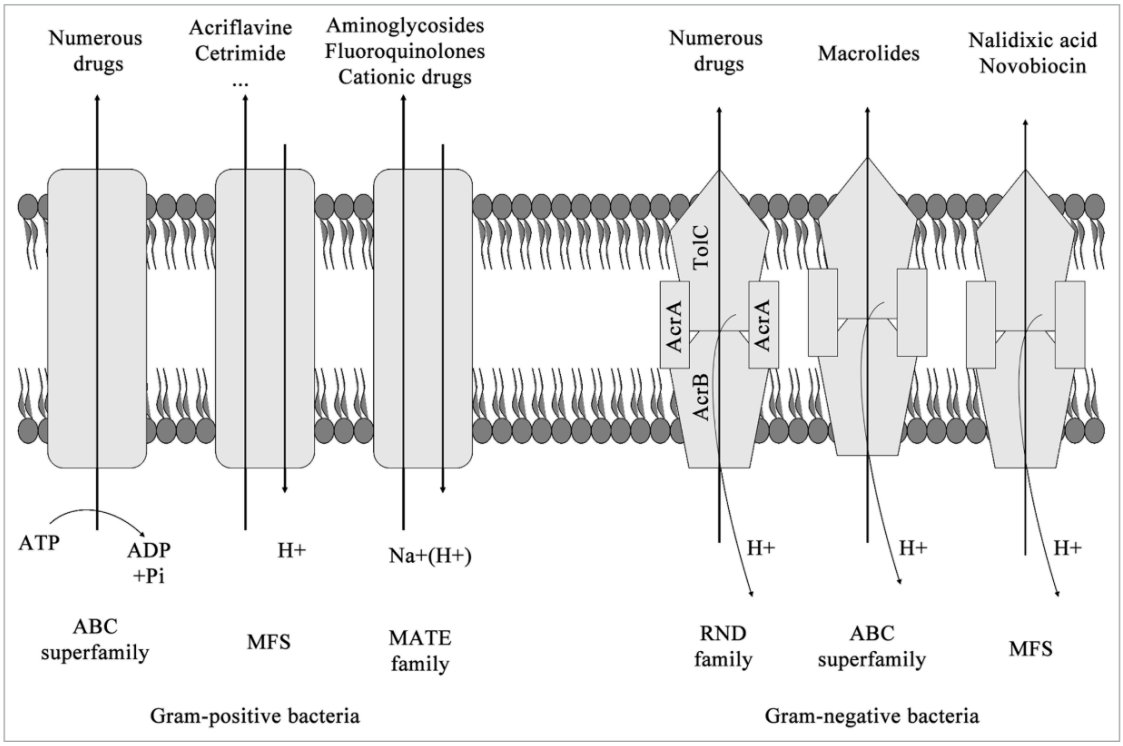

Figure 8 | **Efflux pump families.** | Taken from (Soto, 2013).

As shown in the image above, these groups are the following:

1. ATP-binding cassette (ABC) superfamily.
2. Mayor facilitator superfamily (MFS).
3. Multidrug and toxic compound extrusion (MATE).

4. The small multidrug resistance (SMR) family.
5. The resistance-nodulation-division (RND) superfamily.
6. The drug metabolite transporter (DMT) superfamily.

7.5. Antibacterial resistance transfer

The rising number of antimicrobial resistances detected is not an isolated issue as it is strongly related to resistance transfer. Genes for antimicrobial resistance are usually located in mobile genetic elements (MGE) such as plasmids, integrons and transposons. This transference can occur between bacteria from the same species but also from different genera, becoming a bigger problem.

Metal contamination seems to be favouring the spreading of AMRs as metal and antibiotic resistance genes seem to appear together on the same MGE, especially plasmids. There is a physical linkage of these resistances that make them to be obtained together. As the environmental contamination seems to be rising, this concern does too, since if a bacterium not exposed to an antimicrobial pressure is in contact with heavy metals, it could gain both the resistance to it and to antimicrobials thanks to an MGE. The use of zinc is swine is going to be prohibited soon as it promotes the appearance of new resistances (Ciesinski et al., 2018).

Bacteria use the mechanisms explained above to gain resistance to both heavy metals and antimicrobials. The compounds that share a resistance mechanism are most likely to be co-selected.

Reduction in permeability[b]	As, Cu, Zn, Mn, Co, Ag	Cip, Tet, Chlor, ß-lactams
Drug and metal alteration[c]	As, Hg	ß-lactams, Chlor
Drug and metal efflux[d]	Cu, Co, Zn, Cd, Ni, As	Tet, Chlor, ß-lactams
Alteration of cellular target(s)[e]	Hg, Zn, Cu	Cip, ß-lactams, Trim, Rif
Drug and metal sequestration[f]	Zn, Cd, Cu	CouA

[a]Abbreviations: Chlor, chloramphenicol; Cip, ciprofloxacin; CouA, coumermycin A; Rif, rifampicin; Tet, tetracycline; Trim, trimethoprim.
[b]Includes reduction of membrane permeability to metals and antibiotics.
[c]Includes drug and metal inactivation and modification.
[d]Includes rapid efflux of the metal and antibiotic.

Figure 9 | **This table shows the mechanisms shared by metals and antimicrobials that bacteria use to gain resistance** | Taken from (Baker-Austin et al., 2006).

Plasmids are not essential for the survival of bacteria but add adaptative advantages to them like heavy metal tolerance, and toxin and antimicrobial resistance. They can be transferred to other bacteria by 3 processes: transduction, which is mediated by a bacteriophage; transformation when the genetic elements are transmitted when placed in a medium and pilus-mediated conjugation.

Transposons are DNA sequences that can change their position within the genome. Some of them and the antimicrobials to which they are resistant are Tn05 (bleomycin, kanamycin and streptomycin), Tn21 (spectinomycin, streptomycin and sulphonamides) and Tn4001 (tobramycin, gentamicin and kanamycin).

Integrons are genetic components carrying several ARGs together in the form of gene cassettes (Mohammed et al., 2014).

The transmission of MGEs can be enhanced by reasons like the presence of biofilms, which dense population increases the plasmid dispersal ratio mediated by conjugation (Molin and Tolker-Nielsen, 2003). Some *E.coli* transposons such

as Tn10 and Tn5 cleave thank to recB and recC genes, mutations in these alleles, named texA, can lead to an increased rate of excision (Lundblad et al., 1984). Mutations in transposase, the enzyme responsible for the "cut and paste" of the transposon through the genome, can enhance the transposition of these genes (Baus et al., 2005).

8. Importance of AMR in cattle

We are surrounded by animal products: a large amount of our diet depends on animal protein, not to mention the exposure we have to domestic animals. Pets can also be a reservoir for ARGs, especially when infections are not treated correctly. Some pet infections are treated with "last resort" antibiotics, a special type of antibiotic reserved for lethal infections in humans (Cameron and McAllister, 2016). Not using these antibiotics properly (*i.e.,* subtherapeutic concentration) could lead to an increase in human mortality as the resistances these antibiotics will grow (Smith et al., 2018).

These are some of the reasons why the OHI is becoming important nowadays. It is necessary to emphasise that stopping these AMRs before they become zoonotic, will improve both animal and human health. For that, a collaboration among all the health sciences, especially between the veterinary and human medical professions is necessary (King et al., 2008).

Antibiotics used in veterinary medicine can be classified into 4 different categories (Categorisation of antibiotics used in animals promotes responsible use to protect public and animal health | European Medicines Agency):

- Category A ("Avoid"): includes antibiotics that are currently not authorised in veterinary medicine in the European Union (EU). These medicines may not be used in food-producing animals and may be given to individual companion animals only under exceptional circumstances.
- Category B ("Restrict"): refers to quinolones, 3rd- and 4th-generation cephalosporins and polymyxins. Antibiotics in this category are critically important in human medicine and their use in animals should be restricted to mitigate the risk to public health.
- Category C ("Caution"): covers antibiotics for which alternatives in human medicine generally exist in the EU, but only few alternatives are available in certain veterinary indications. These antibiotics should only be used when there are no antimicrobial substances in Category D that would be clinically effective.
- Category D ("Prudence"): includes antibiotics that should be used as first line treatments, whenever possible. These antibiotics can be used in animals in a prudent manner. This means that unnecessary use and long treatment periods should be avoided, and group treatment should be restricted to situations where individual treatment is not feasible.

Tables 1 and 2 show the antibiotic classes belonging to each group and examples of active ingredients for each class. This information is useful to understand the resistances found in our samples (Table 3). We need to focus on finding whether or not we have resistances to antibiotics belonging to the category A or B. In case

of being found, it would be important to understand how those ARGs arrived in the rumen microbiota.

AMEG Categories	Antibiotic class, subclasses	Example of antibiotic(s)
Category A (*"Avoid"*)	Amdinopenicillins	mecillinam, pivmecillinam
	Carbapenems	meropenem, doripenem
	Other cephalosporins§ and penems (ATC code J01DI), including combinations of 3rd-generation cephalosporins with beta-lactamase inhibitors	ceftobiprole, ceftaroline, ceftolozane-tazobactam, faropenem
	Glycopeptides	vancomycin
	Glycylcyclines	tigecycline
	Ketolides	telithromycin
	Lipopeptides	daptomycin
	Monobactams	aztreonam
	Oxazolidinones	linezolid
	Penicillins: carboxypenicillins and ureidopenicillins, including combinations with beta-lactamase inhibitors	piperacillin-tazobactam
	Phosphonic acid derivates	fosfomycin
	Pseudomonic acids	mupirocin
	Rifamycins (except rifaximin)	rifampicin
	Riminofenazines	clofazimine
	Streptogramins	pristinamycin, virginiamycin
	Sulfones	dapsone
	Drugs used solely to treat tuberculosis or other mycobacterial diseases	isoniazid, ethambutol, pyrazinamide, ethionamide
	Substances newly authorised in human medicine following publication of the AMEG categorisation.	To be determined.
Category B (*"Restrict"*)	Cephalosporins: 3rd- and 4th-generation, except combinations with beta-lactamase inhibitors	ceftiofur, cefovecin, cefquinome
	Polymyxins	colistin, polymyxin B
	Quinolones: fluoroquinolones and other quinolones	enrofloxacin, ciprofloxacin, ofloxacin, oxolinic acid
Category C (*"Caution"*)	Aminoglycosides (except spectinomycin)	streptomycin, gentamicin
	Aminopenicillins in combination with beta-lactamase inhibitors	amoxicillin-clavulanic acid
	Amphenicols	florfenicol, thiamphenicol
	Cephalosporins: 1st- and 2nd-generation, and cephamycins	cefalexin, cefapirin
	Macrolides (not including ketolides)	tylosin, tulathromycin
	Lincosamides	clindamycin, lincomycin
	Pleuromutilins	tiamulin, valnemulin
	Rifamycins: rifaximin only	rifaximin
Category D (*"Prudence"*)	Aminopenicillins, without beta-lactamase inhibitors	amoxicillin, ampicillin
	Cyclic polypeptides	bacitracin
	Nitrofuran derivatives*	furazolidone
	Nitroimidazoles*	metronidazole
	Penicillins: Anti-staphylococcal penicillins (beta-lactamase-resistant penicillins)	cloxacillin
	Penicillins: Natural, narrow spectrum penicillins (beta-lactamase-sensitive penicillins)	benzylpenicillin, phenoxymethylpenicillin
	Aminoglycosides: spectinomycin only	spectinomycin
	Steroid antibacterials*	fusidic acid
	Sulfonamides, dihydrofolate reductase inhibitors and combinations	sulfadiazine, trimethoprim
	Tetracyclines	oxytetracycline, doxycycline

Table 1 | **Antibiotic class and examples of them by category. Taken from** (European Medicines Agency (EMA), 2019)**.**

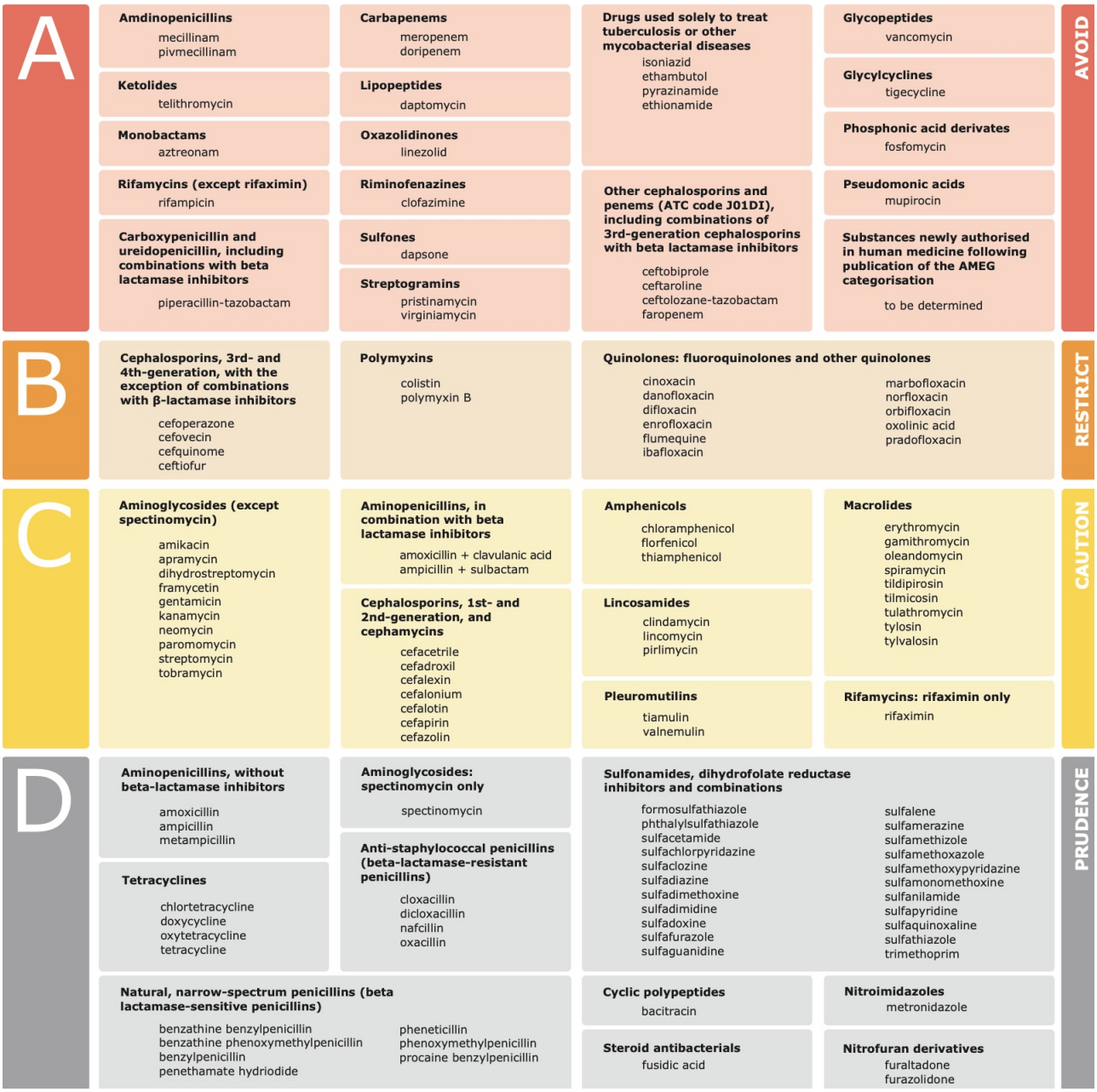

Table 2 | **Antibiotic class and their active ingredients by risk group. Taken from** (EMA, 2019)**.**

9. The rumen microbiota and its role in AMR

Rumen microbiota produces 70% of daily energy required by the ruminants (Bergman, 1990), what means that controlling the microbiota and the host-microbiome interactions could help to address issues as feed conversion ratio, methane emissions, and reduce the number of AMRs.

Ruminants are responsible for 5% of the total methane emissions in Spain (Ministerio para la Transición Ecológica, 2019), being controlled mostly by methanogenic archaea as it is a by-product of their metabolism. This opens a new field of study where selecting or manipulating the microbiome will allow us to reduce the ruminant methane emissions (Roehe et al., 2016a).

This first stomach usually functions with solid intake and presents physical and functional differences between fully developed adults whose microbiota carry out microbial fermentation and pre-ruminants that only feed on milk (Heinrichs, 2005). There are several factors that alter the microbiome composition such as age, feed, diet, location, host species, breed and season (Malmuthuge and Guan, 2017).
Adult microbiota is resistant to perturbations, while pre-ruminants microbiota seems to have greater plasticity and be easier to modify, suggesting that adult microbiota is more difficult to manipulate with elements like microbiota transplants, prebiotics or probiotics (Weimer, 2015). Also, the capability to modify early life microbiota to have long-term results. This seems to indicate that modifying young calves microbiota would help to determine the composition of their adult ruminal microbiota, what will aid to produce cows with improved feed efficiency, fewer methane emissions (Abecia et al., 2013, 2014) and less susceptibility to subacute ruminal acidosis (SARA) (Chen et al., 2012).
There are no current studies in cattle, but in young goats the impact of the early rumen microbiota on metabolism (Morgavi et al., 2015), methane production (Hegarty et al., 2008; Abecia et al., 2013) and microRNAs expression (Liang et al., 2014) is under research. Dietary supplements in young goats (from birth to 3-month-old) change the composition of the rumen microbiota lasting 3 months from the day the supplementation was stopped (Abecia et al., 2013). It has also been studied (Hegarty et al., 2008) that privation of protozoa acquisition in early stages of life changed the microbial composition, urine metabolites and fermentation in adult stages. This seems to indicate that early manipulation of microbiota will lead to modifications in adult microbiota composition and therefore in adult phenotype. Manipulating the microbiota in calves could be more successful than manipulating it in adults.
Adaptation has resulted in a wide variety of rumen sizes, morphological and physiological characteristics. This included passage rates, which allows ruminant species to exploit a huge range of feed types. These host adaptations could also play a role in regulating rumen microbiota (Russell and Rychlik, 2001).

9.1. Ruminal microbiota composition

9.1.1. Bacteria

Bacteria are the most abundant microorganisms represented by over 200 species with a concentration of 10^{10}-10^{11} cells per millilitre, constituting more than 70% of the microbial communities (Mcsweeney and Mackie, 2012; Pitta et al., 2016a). Their growth depends on the substrates available, the energy requirements and resistance to toxic metabolic end-products.
Diet determines the prevalence of certain bacterial species. Gram-negative bacteria grow more with forage diets, and Gram-positive with grain diets like *Lactobacillus.* In high forage diets, bacteria are responsible for the degradation of cellulose and hemicellulose. *Fibrobacter succinogenes* and *Ruminococcus albus* are the most desirable degraders (Koike and Kobayashi, 2009).

Only 20% of bacterial rumen species can be cultured in laboratories, therefore 16S rRNA gene techniques are becoming more important. With them, we can analyse the composition of a certain population, predict their functionalities or enumerate the microbes in an ecosystem (Matthews et al., 2019).
67.1% of all bacterial composition is composed of seven groups (Henderson et al., 2015b; Weimer, 2015): *Prevotella, Butyrivibrio, Ruminococcus, Lachanospiraceae, Ruminococcaceae, Bacterioidales* and *Clostridiales*. These groups are the "core bacterial microbiome" (*figure* 10).

9.1.2. Methanogenic archaea

Rumen archaea are strictly anaerobic but with a broad variety of metabolisms. Archaea are the only microorganisms capable of producing methane in the rumen and are found in concentrations similar to 10^{6}-10^{8} cells/ml, being fewer than 4% of the microbial community (Lin et al., 2006)
Methanobrevibacter gottshalkii and *Methanobrevibacter ruminantium* are the two largest clades found in almost all samples, computing for 74% of all ruminal archaea (Henderson et al., 2015b; Weimer, 2015).
Methanogens usually colonise protozoa, enhancing methane formation in the rumen (Newbold et al., 1995).

9.1.3. Ciliate protozoa

They digest around 30-40% of the ingested fibre with a concentration of 10^{4}-10^{6} cells/ml. Protozoa are active in lipid hydrolysis and produce hydrogen in their hydrosomes (Mcsweeney and Mackie, 2012). *Entonidium* and *Epidinium* genera are the ones with larger prevalence in high grain diets, representing 54.7% of protozoal sequence data (Weimer, 2015). They degrade starch into an iodophilic storage polymer. This degradation is mediated by amylases, glucosidases and debranching enzymes.

9.1.4. Bacteriophages

Bacteriophages are bacterial viruses found in 10^{7}-10^{9} particles/ gram of ingesta (Klieve et al., 1996). It is believed that they intervene in the evolution of bacterial systems facilitating HGT for both bacteria and protozoa (Berg Miller et al., 2012).

Microorganism	Kingdom	Location	Related to
Prevotella spp.	Bacteria	Rumen microbiota	Metabolism of proteins and peptides
Firmicutes[a]	Bacteria	Rumen microbiota	Milk fat yield, impaired feed conversion, residual feed intake
		Oral microbiota	High relative abundance in oral microbiota before calving
Bacteroidetes[a]	Bacteria	Rumen microbiota	Milk fat yield, impaired feed conversion, residual feed intake
		Reproductive tract microbiota	Postpartum fever in primiparous cows, birth of twins, assistance at parturition, metritis
Butyrivibrio	Bacteria	Rumen microbiota	Degradation of hemicellulose and butyrate production
Methanobrevibacter spp.	Archaea	Rumen microbiota	Methane production
Fusobacteria	Bacteria	Reproductive tract microbiota	Postpartum fever in primiparous cows, birth of twins, assistance at parturition, metritis
Proteobacteria	Bacteria	Reproductive tract microbiota	High abundance in uterus and vagina
Klebsiella spp.	Bacteria	Udder microbiota	Mastitis
Streptococcus uberis	Bacteria	Udder microbiota	Mastitis
Trueperella pyogenes	Bacteria	Udder microbiota	Mastitis
Escherichia coli	Bacteria	Udder microbiota	Mastitis

[a] The ratio of Bacteroidetes/Firmicutes is considered as a predictor of the metabolism of the host.

Figure 10 | **Summary of the most important microorganisms with association with digestion, reproduction and udder health** | Taken from (González-Recio et al., 2019).

9.1.5. Amoeba

Their role is still unknown. It has been studied that they ingest bacteria through phagocytosis and there is some indication of endosymbiosis (Lambie et al., 2015). *Campylobacter jejuni* invades *Acanthamoeba polyphaga* and replicates in its vacuoles (Olofsson et al., 2013) and has been proved that *C. jejuni* its resistance to acid by co-incubation with amoebas (Axelsson-Olsson et al., 2010) allowing this bacterium to tolerate better the rumen conditions. *C. jejuni* and *C. fetus* have important effects on cow fertility and immunity. For this reason, it would be of great interest to study whether *C. fetus* can co-incubate with other amoeba species or not, as it is responsible for bovine campylobacteriosis, a venereal disease that causes infertility in females, increasing the number of services needed for conception or causing late-term abortions (Hoffer, 1981).

9.1.6. Fungi

Neocallimastigomycetes is the most prevalent class in the rumen, consisting of 6 genera: *Anaeromyces, Caecomyces, Cyllamyces, Neocallimastix, Orpinomyces* and *Piromyces*. Fungi concentration fluctuates between 10^{3}-10^{6} zoospores/ml (Matthews et al., 2019). All ruminal fungi are anaerobic which required a revaluation of all previous information claiming that fungi were strictly aerobic (Krause et al., 2013). This adaption is most likely conceived by horizontal transfer of bacterial genes.

Fungi degrade vegetal walls producing H_2 which is used as a substrate by other microorganisms. They produce high concentrations of cellulase and hemicellulase as well as xylanase to break down xylan. This makes them the initiators of digestion (Akin and Borneman, 1990).

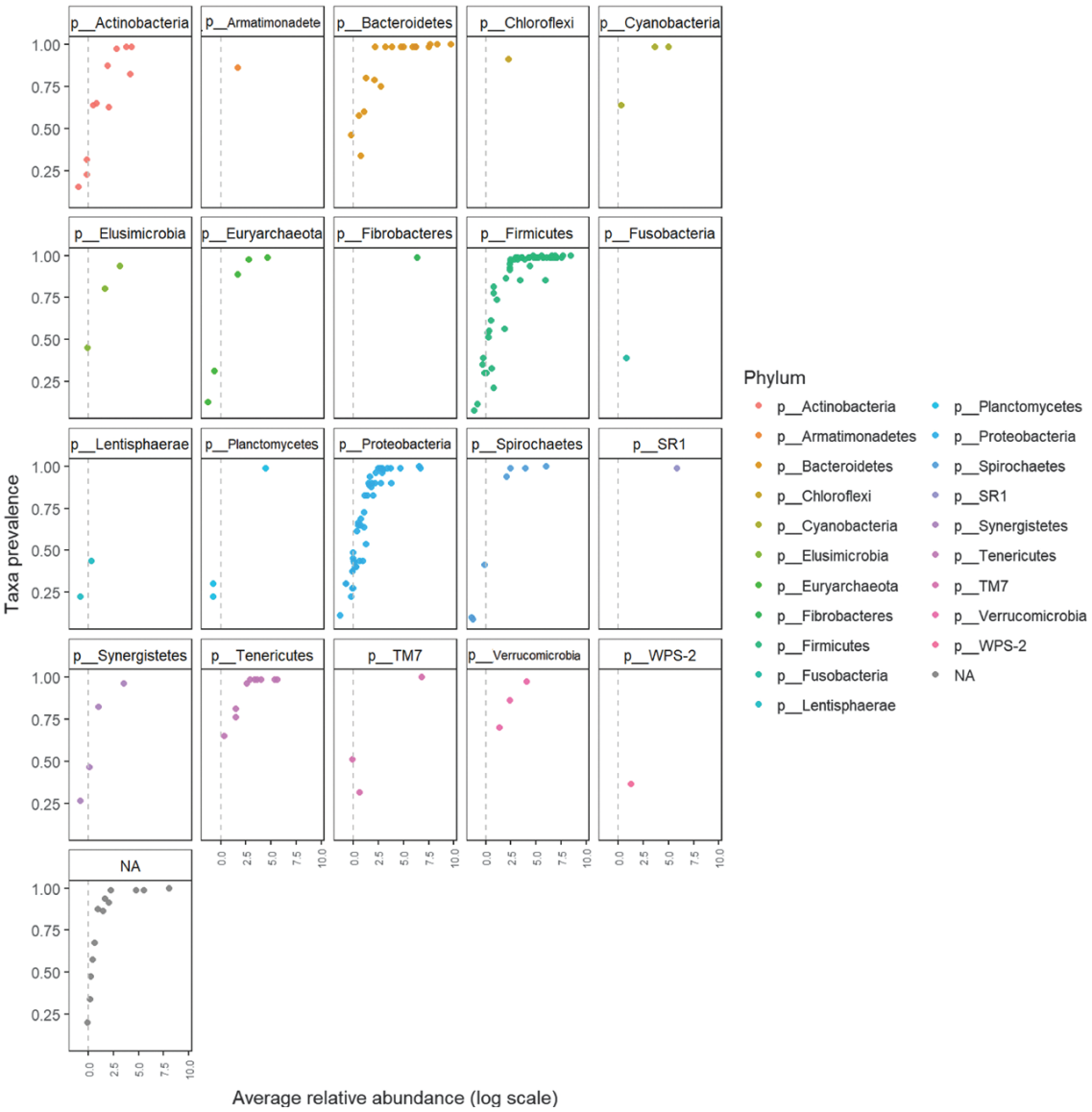

Figure 11 | **Prevalence of different phyla in the rumen microbiota composition** | Taken from (González-Recio et al., 2019).

9.2. Role of the ruminal microbiota in AMR

The microbiome acts as a modulator for the phenotypic expression of several traits such as methane emissions, health status and feed efficiency (Zhang et al., 2007). It was not until a few years ago that we started to study the bacteria-host interaction and rather than the laboratory-isolated bacteria. Especially, more attention has been placed on the interaction between microbes and diet (Mohammed et al., 2014), composition across host, environment and age (Henderson et al., 2015b), methane emission (Yáñez-Ruiz et al., 2010) and as predictor of complex traits (Ross et al., 2013).

Prevotella, a genus responsible for the metabolism of peptides and proteins, have a strong relationship with *Lachnospiraceae*, suggesting that an increase in Relative Abundance (RA) is related to decrease *Firmicutes* and *Lachnospiraceae*. *Prevotella* and *Paraprevotella* metabolise proteins and carbohydrates, synthesise

de novo peptides and use products of cellulose degradation from other bacteria metabolism (Lou et al., 1997; Gonzalez-Recio et al., 2018).
Methanobrevibacter and *Methanosphaera* use H_2, CO_2 and other bacterial by-products to synthesise methane. These two genera are the most responsible for CH_4 emissions. Decreasing the biodisponibility of hydrogen could reduce the methanogenic archaea population, some flavonoids compounds are being used for that (Seradj et al., 2014).
Dasytricha, Diplodinium, Entodinium, Eremoplastron, Isostricyha and *Trichostomatia* are the most abundant protozoa genera. It seems that they share strong interrelationships, playing a role in digestion and fermentation of feed components. They use ruminal O_2 and produce the hydrogen that methanogenic archaea use to produce methane (Newbold et al., 2015). *Isostrichya* and *Dasytricha* use soluble sugar for their metabolism, *Entodinium* degrades cellulose from small plants and *Polypastron* feed on large fibres from the rumen fluid. Using these compounds results in the production of acetate, butyrate, lactate, CO_2 and H_2 a by-product that can are converted in methane by methanogenic archaea (Jouany, 1991). Protozoa interact with other rumen microbes as they use bacteria as protein source. Feed efficiency or methane yields and some of the traits whose heritability could be explained by a host genetic effect on the RA of these microorganisms (Pryce et al., 2014; Gonzalez-Recio et al., 2018).

9.3. Most relevant ARGs

In cattle, only antimicrobial resistances and not genes associated to antimicrobial resistances have been described in actual animals (*figure* 12), we know that, with regards to human health, the most important bacteria-antibiotic binomials are *Campylobacter spp*-Quinolone and macrolide resistance and *Salmonella spp.*-Quinolone and 3rd and 4th generation of cephalosporin (Engberg et al., 2001; Jeon et al., 2019).
Ruminal ARGs could seem of a lowest importance as they cannot directly jump to humans, other animals or the soil. But it is important to determine if the same ARGs are present in saliva, ruminal fluid or faeces. During the cud the feed goes back to the mouth, where ruminal AMR bacteria could pass their ARGs to the oral microbiota. Same happens for the faecal microbiota.
In ovine rumen the most prevalent genes are the presented in figure 13, so we can expect similar results.

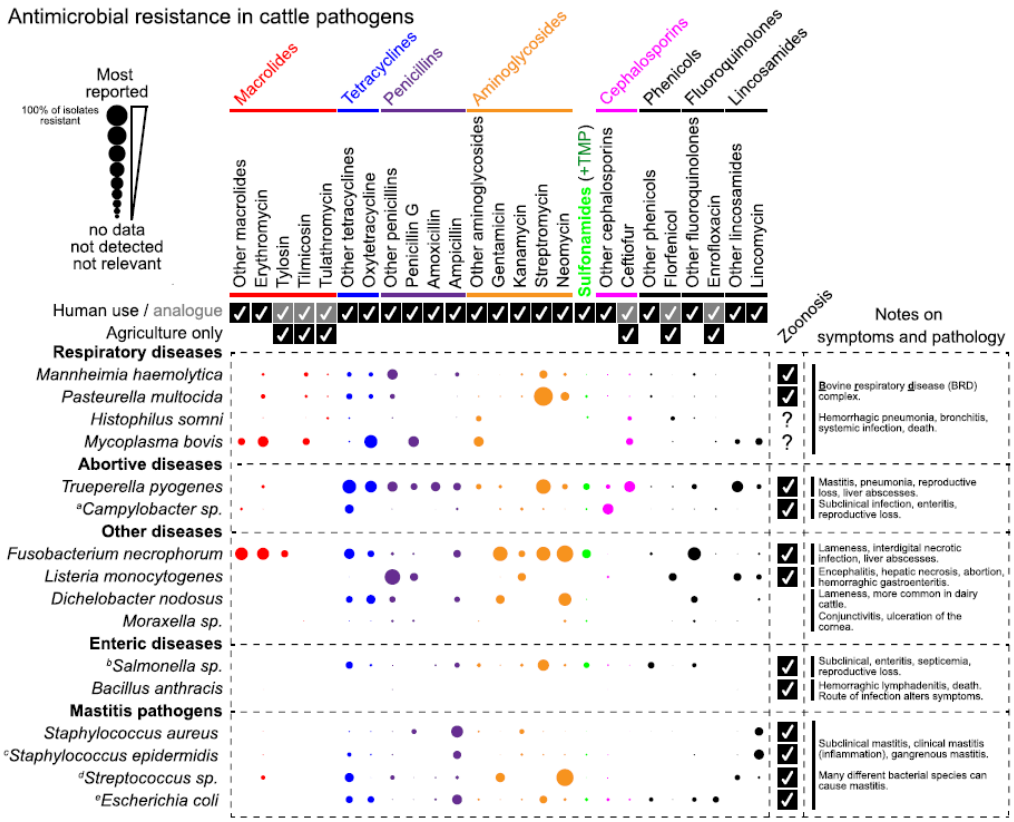

Figure 12 | **This table shows the most used antimicrobials in cattle, whether they have been used in human diseases and whether the disease is zoonotic or not.** | (Cameron and McAllister, 2016).

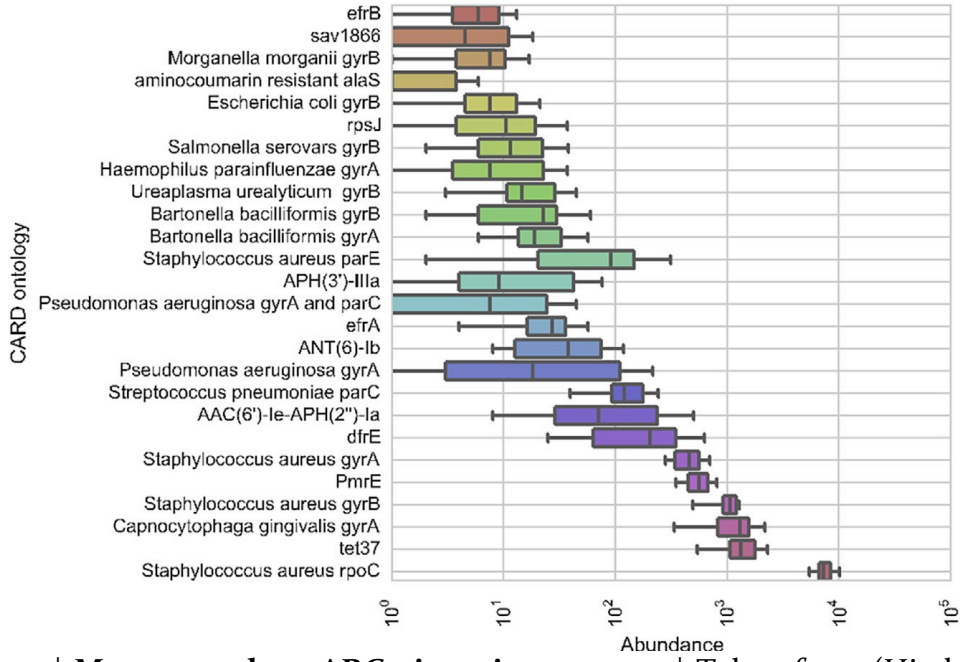

Figure 13 | **Most prevalent ARGs in ovine rumen.** | Taken from (Hitch et al., 2018).

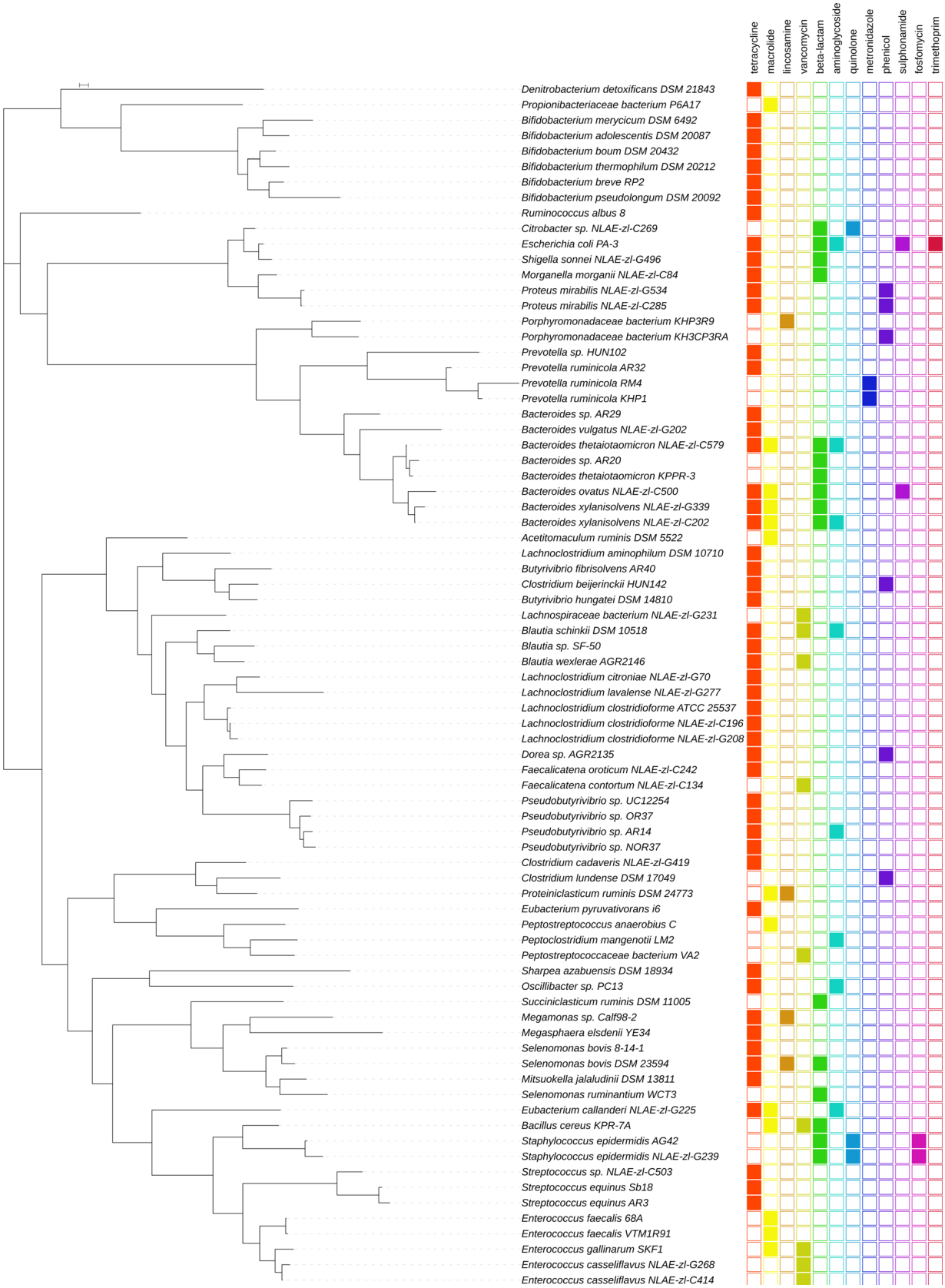

Figure 14 | **Distribution of AMR bacteria in ruminal microbiota.** | Taken from (Sabino et al., 2019).

10. The udder microbiota and its role in AMR

The teat canal has strong barriers against microbes but there are some species than can surpass it and proliferate in the intrammary ecosystem (González-Recio et al., 2019). The udder microbiota is extremely related to the health of the cattle, as a healthy one would avoid the growth of pathogens that can create a dysbiosis. A low microbial diversity is associated with severe diseases like mastitis (Li et al., 2018).

Pseudomonas, Acinetobacter, Loctococcus, Propionibacterium, Aeribacillus, Lachnospiraceae, Staphylococcus, Streptococcus and *Faecalibacterium* are the most prevalent genera in the udder microbiome of healthy cows, while *Trueperella pyogenes, Streptococcus uberis, Escherichia coli* and *Klebsiella spp, among others* are associated with mastitis (Li et al., 2018).

Mastitis is the most common and costly disease affecting dairy cattle and it is responsible for the majority of antibiotic use (Mitchell et al., 1998). It is an intramammary infection (IMI) caused mostly by staphylococci, streptococci and Gram-negative bacteria although more than 135 microorganisms can be a cause of this disease (Bradley, 2002). In some farms outside the European Union, cows are given antibiotics to lower the risk of suffering from this disease (Ruegg and Petersson-Wolfe, 2018), caused by both Gram-positive and negative bacteria (Erskine et al., 2002). The actual treatment is an intramammary injection of penicillin (Raymond et al., 2006). We would expect an increase in the prevalence of penicillin-resistant bacteria. In Europe, penicillin-resistant *Streptococcus aureus* has a prevalence of 3-46%, whilst the resistance to other antimicrobials is lower than 10% (Hendriksen et al., 2008).

Increasing milk hygiene is one of the control measures taken to reduce the prevalence of pathogens. Nevertheless, environmental streptococci have become more prevalent and the reduction in subclinical mastitis has not been followed by a reduction in the clinical cases (Pitkälä et al., 2004).

Antimicrobials usage is higher in dairy cattle than in beef cattle, so it is not unexpected that the prevalence of AMRs in dairy cattle is larger than in beef. As stated previously, the use of antibiotics in the dairy industry is mainly related to mastitis treatment. Still, there are studies showing that mastitis-causing pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* have limited ARGs (Erskine et al., 2002). On the other hand, there are other studies showing that there is no correlation between the prevalence of AMR bacteria and the antimicrobial use either not being responsive to the use of antimicrobials or being in a large quantity even when no antimicrobials are used (Call et al., 2008).

(Erskine et al., 2002) found that while *Streptococcus uberis* had become more resistant to penicillin, they have also become more susceptible to sulfa-trimethoprim, gentamicin, pirlimycin and oxacillin. Mastitis treatment is a significant proportion of total antimicrobial usage, which makes AMR limited in mastitis pathogen when compared to enteric organisms.

In infected cows, coagulase-negative staphylococci (CNS) and *Corynebacterium bovis* were the pathogens most frequently found (9.1% and 7.3% respectively), as well as *Staphylococcus aureus* (5.7%) and *Streptococcus uberis* (1.0%). *Streptococcus agalactiae* was found in 29% of herds in Germany. The prevalence

of most pathogens is higher in older cows, but CNS are found in larger proportion in primiparous cows (Tenhagen et al., 2006).
Around 70% of the times, cows with clinical mastitis are transferred to a sick cow pen. Cephalosporins are the first-choice treatment followed by β-lactamase–resistant penicillins and conventional penicillin. *Staphylococcus aureus* resistance to ampicillin increases after the first lactation (Sol et al., 2000). Most of the farmers recognise treating their cows 3 to 4 times per case of mastitis. It would be interesting to investigate whether there is a correlation between both events.
Two relevant aspects must be highlighted. The first one is a decrease in cure rates after a first treatment (Sol et al., 2000). The second one is, as pointed previously, the transmission of resistant bacteria to the food chain (Tenhagen et al., 2006a). The problem comes when clinical infections are diagnosed as subclinical cases or cannot be diagnosed as they are latent infections and pass to the milk.
CNS bacteria is the most prevalent in healthy cows, especially in primiparous ones. *Staphylococcus aureus* was the predominant pathogen proportionally increasing its prevalence with age and stage of lactation. *Streptococcus uberis* was the most frequent environmental pathogen found, increasing its prevalence during lactation in older cows. Ampicillin-resistant isolates of *Staphylococcus aureus* was found in lower proportion in primiparous cows (Tenhagen et al., 2006b).
Currently, there are some phytochemicals under research for their ability to treat several diseases. *Allium sativum* (Dilshad et al., 2010), *Zingiber officinale* (Poeloengan, 2011), *Allium cepa* and *Trachyspermum ammi* (Fujisawa et al., 2009) are being used to treat mastitis caused by antimicrobial-resistant pathogens.
Most antibiotics in dairy farms are used to treat mastitis. The level of antibiotic-resistant *S. aureus, Str. uberis* and *Str. dysgalactiae* in adults and *E. coli* and *Salmonella enterica* in calves is rising (Lacy-Hulbert and Blackwell, 2015).
Even though mastitis isolates of *Streptococcus spp.* and *S. aureus* show no evidence for an increase in AMR, there are studies trying to understand where the selection for ARGs happens, and it seems that Methicillin-resistant *Staphylococcus aureus* (MRSA) has been selected in the nose and Extended Spectrum Beta-Lactamase (ESBLs) producing Enterobacteriaceae has happened in the gut (Leuenberger et al., 2019).
Lameness and uterine problems like metritis and placenta retention are other prevalent diseases that require antibiotic treatment. Lameness is mostly caused by *Dichelobacter nodosis* and *Fusobacterium necrophorum,* these bacteria have genes (*fimA* and *lktA* respectively) that encode for leukotoxins. (Bennett et al., 2009). This disease is more common in early lactation and old cows are more likely to suffer from it than young ones (Warnick et al., 2001). Severe cases are treated with a bandage soaked in oxytetracycline hydrochloride and lincomycin or spectinomycin (Shearer, 1997). Hooves baths carry frequently antibiotics to prevent this issue, making them a possible focus of ARMs transmission (Holzhauer, 2017).
Metritis is mainly caused by *Trueperella pyogenes* but can also be caused by *Salmonella spp., Listeria spp.,* and *Coxiella burnetii.* Isolates of *T. pyogenes* show resistance to all the antibiotics for which they were tested in (Santos et al., 2010)'s study. These isolates presented high level of resistance to amoxicillin (56.9%),

ampicillin (86.1%), chloramphenicol (100%), florfenicol (59.7%), oxytetracycline (54.2%), penicillin (86.1%) and tetracycline (50%). With regards to multiresistant bacteria, 95.8% of the isolates were resistant to at least 2 of the antimicrobials used, and 88.9% were resistant to at least 3 antimicrobials. They found that no isolate was resistant to all the 9 antibiotics, but 5.6% of them, were resistant to 8 ATBs. These results are worrying, as they indicate that a lot of bacteria are multiresistant to the most used ATBs to treat mastistis.
There are several factors other than antimicrobial resistance that determine the severity of the infection such as the ability of the microorganism to form biofilms, host-pathogen interactions and cattle health.

10.1. Most relevant ARGs

As mastitis is the main reason for antimicrobials use in dairy cattle, we will point out the ARGs present in bacteria causing this disease (Gentilini et al., 2002; Qu et al., 2019):

- β-lactam resistance genes: *blaZ* and *mecA*.
- Tetracycline resistance genes: *tetK*, *tetL*, *tetM*, and *tetO*.
- Aminoglycoside resistance genes: *aacA-aphD*, *aadD*, and *aphA3*.
- Macrolides, lincosamides, and streptogramin B; MLSB resistance genes: *ermA*, *ermB*, *ermC*, *ermT*, *msrA*, *mphC*, and *lnuA*.
- Trimethoprim resistance genes: *dfrG* and *dfrK*.
- Vancomycin resistance genes: *vanA* and *vanB*.

11. The faecal microbiota and its role in AMR

The faecal microbiota changes during the first 10 weeks of live of the cows as it is shown in figure 15.

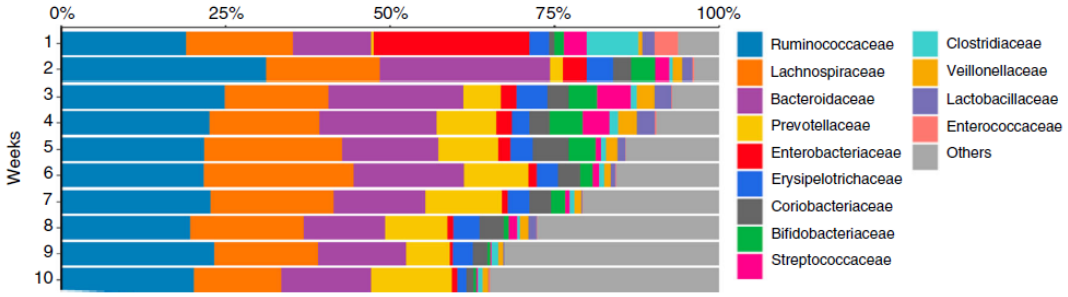

Figure 15 | **Relative abundance of bacterial families over time** | Taken from (Liu et al., 2019).

Shedding is a problem if we think about using faeces as manure, as it will carry more AMRs, which could pass ARGs to soil or plant bacteria by horizontal gene transfer. Faecal bacteria shedding is also a problem to be aware of. It was proved that cold stress, heat stress, overcrowding, intermingling and poor sanitation are some of the conditions that increase the rate of shedding (Moro et al., 2000; Mathew et al., 2003). This event can become a problem if the animal carries a high load of AMRs. It is suggested that bacterial shedding is also influenced by age, as it is superior in young calves than in older cows being a possible bias in the interpretation of the AMRs analysis. This phenomenon is not necessarily

caused by the administration of antibiotics as it can also happen because of neonatal-adapted bacteria, especially *E. coli.* (Khachatryan et al., 2004a).
Another important issue is the fact that stressed animals shed more AMR and pathogenic bacteria like *E. coli*. Stress is not intrinsically related to AMR, but it will accelerate faecal mobility. This can lead to a horizontal transmission problem; might it be within bacteria or zoonotically. (Moro et al., 2000; Mathew et al., 2003).

11.1. Presence of AMR bacteria in organic farms

Organic farms have a very restrictive normative with regards to the use of antimicrobials and for that, they can be used as a comparison to those conventional farms which use therapheutic antibiotics.
Even though it seems to be a relation between organic farms and lower presence of AMR bacteria, resistant bacteria were found on organic farms soil after years of antimicrobial-free management, proposing that there are other factors besides antimicrobial use involved in the long-term persistence. (Walk et al., 2007) proved that point and concluded that the ampicillin-resistant population on conventional dairies was a consequence of antimicrobial use, whilst tetracycline-resistant bacteria presence is unrelated to antimicrobial use. In the same paper, they give evidence of the fact that in organic and conventional dairies *E. coli* do not group under the same phylogenetic branch, which suggests that different strains have different ways of assimilating genes.
Despite having found higher levels of AMR bacteria in convectional farms, faecal isolates proved that most of *E. coli* and *Salmonella* species are susceptible to several antibiotics (Lundin et al., 2008).
In faecal samples, those of conventional dairies present *Salmonella* resistant to streptomycin or sulphonamides (Sato et al., 2005), higher prevalence of multiple-resistant *E.coli*, no differences in *Campylobacter* (Halbert et al., 2006) and higher resistance to tetracycline than those of organic farms.

11.2. Use of antimicrobials at subtherapeutic concentration

Administering the antibiotics during less time than recommended or not using the exact dose causes the appearance of new resistance, as it is easier for bacteria to be positively selected.
The number of genes coding for antimicrobial resistance can be increased by the addition of antibiotics in the food. The subtherapeutic concentration of these drugs helps to the development of new resistances and the persistence of the existing ones. (Alexander et al., 2011) conducted an experiment where they studied the effects of the administration at a subtherapeutic concentration of chlortetracycline, chlortetracycline plus sulfamethazine and tylosin on *tet, sul* and *erm* resistance genes and bacterial shedding in faeces. They found out that the concentration of 16S-rRNA genes increased until day 56 of the treatment. To that point, the concentration started to decrease, being by day 175 the same as day 7. This means that a subtherapeutic dose would increase the concentration of resistance genes and not reducing the bacterial population. The concentration of tetracycline resistance genes *tet(*B*), tet(*C*), tet(*M*)* and *tet(*W*)* was affected by

treatment and time. t*et(*B*)* concentration was increasing until day 42 and decreased to normal levels by day 112. Similar results following the same pattern of increase-decrease were observed for the rest of the genes.
The behaviour of sulfonamide resistance genes remained similar to tetracycline ones, showing an increase in the first four months of treatment and then a decrease in its concentration.
Erythromycin resistance genes were also affected by the type of treatment and time of exposure. The results were similar to those of the genes noted before. An increase of the values by day 85 and regularisation of the values by day 175.

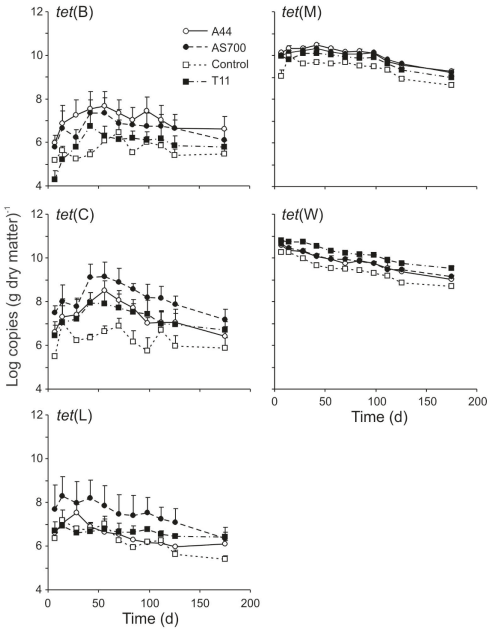

Figure 16 | **Persistence of tetracycline resistance genes** | We can see an increase in the concentration during the first days but falling to normal levels or lower from day 100-120. Taken from (Alexander et al., 2011).

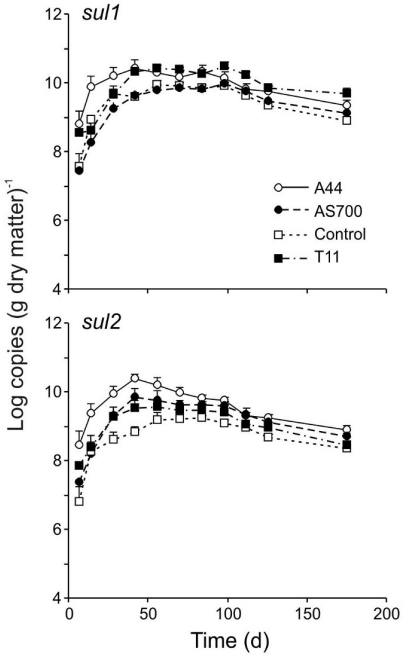

Figure 17 | **Persistence of sulphonamide resistance genes.** | We can see an increase in concentration until day 50 and then the decrease mentioned before. Taken from (Alexander et al., 2011).

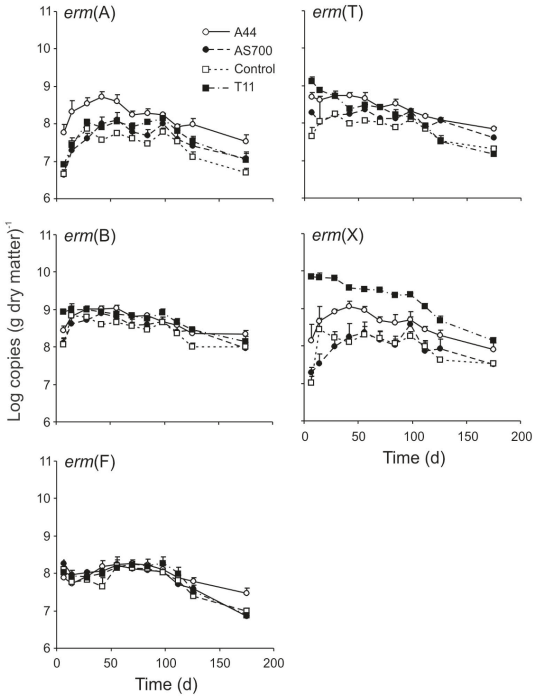

Figure 18 | **Persistence of erythromycin resistance genes** | Taken from (Alexander et al., 2011).

Time of exposure has a greater effect than the type of antibiotic administered on bacterial ecology. Presence of tetracycline, tylosin or sulphonamide alters the microbiota. Bovine faeces can serve as a reservoir for long periods of time for ARGs. The genes *tet(*L), *tet(*W), *erm(*F) and *erm(*T) did not increase on faecal deposits over time, in fact, declined.

Subtherapeutic usage of drugs works as a selective pressure for AMRs (Alexander et al., 2008). Around 75% of the antimicrobials used in livestock is excreted in urinal and faecal waste (Chee-Sanford et al., 2009). This excess has been proved to have limited selective pressure on AMRs (Alexander et al., 2011). In the same study, it was proved that the selective forces acted with more intensity in cattle guts than in soil, but this does not mean that faeces used as manure are not a focus of ARG transmission.

Studies such as (Berge et al., 2005) proved associations but not causal links between antimicrobials and development of resistances at host level. In this research, florfenicol was injected via skin in feedlot steers, showing a prevalence if faecal *E. coli* resistant to chloramphenicol. The same thing happened with ceftiofur. Two weeks after the injection, the levels returned to normal values close to zero (Lowrance et al., 2007). This shows that antimicrobial use can lead to a higher prevalence of AMR in faeces, although the effect is not necessarily permanent.

(Alexander et al., 2008) carried an experiment in which they fed antimicrobial to cattle in the same concentrations as they are given in conventional farms for prophylactic and growth purposes. The first faecal samples at the arrival of the animals demonstrated that tetracycline-resistant bacteria were present in approximately 40% of the herd. They had 5 different groups, but in the one fed for tetracycline and sulphonamide, it was observed an increase in tetracycline-

resistant bacteria. Some other groups were supplemented with ampicillin and gentamicin, but the prevalence of bacteria resistant to these antibiotics was uncorrelated. Ampicillin resistance increased by cause of clonal expansion of a strain that outcompeted the rest of them. This points out that not only antimicrobial resistance genes (ARGs) but also other bacterial fitness traits can play a role in the dissemination and emergence of antimicrobial-resistant bacteria in livestock as well as commensalistic bacteria such as *E. coli, Enterococcus spp.* and *Streptococcus spp.*

It is also remarkable the difference of rearing between beef and dairy calves. While beef ones are raised on rangeland, dairy calves are raised intensively and treated with multiple antimicrobials to cure more prevalent pathologies like diarrhoea and pneumonia (Call et al., 2008). It is reasonable to think that beef calves have a lower prevalence of AMRs as reported by (Davis et al., 2007). They isolated *Salmonella enterica* serovar Dublin and found that those samples from dairy calves were more resistant to antimicrobials. This can be explained because S. Dublin is a serovar specifically adapted to cattle, having obtained this adaptation because of selective pressure and not by dissemination. When using adult populations, this difference blurs and their isolates have similar proportion (Parveen et al., 2006).

Selection of AMR can lead to the co-selection of an unrelated trait (Chen et al., 2008). If the linkage occurs with a positive trait, it is common that the ARG stays conserved even without selection pressures for antimicrobial resistance. Experimental competition studies (Khachatryan et al., 2004a; b, 2006a) were performed demonstrating that sulphonamide and tetracycline-resistant *E.coli* (SSuT) had a growing advantage over regular *E. coli* in rich media and calves younger than 3 months but it disappeared in heifers older than 11 months.

There are some ideas that could explain this phenomenon like an antimicrobial selection pressure acting as a maintainer for the high prevalence of SSuT strains. Another approach could be that SSuT traits are linked to another trait that confers an advantage. The main result of these studies was that the prevalence of SSuT was linked to young dairy calves.

With all these hints, we can conclude that there is an antimicrobial selection pressure in cattle. The use of antimicrobials leads to an increase in the prevalence of AMR bacteria that will disappear once the selection pressure is taken away (Call et al., 2008). If the ARGs imply a physiological added cost, we would expect a decrease in the number of AMR organisms. If not, we would expect a reduction in the prevalence in the face of natural turnover of clonal types at the level of individual animals (Jenkins et al., 2003). It is possible that the AMR traits get linked to other traits that suppose a genetic advantage in a specific niche. This type of linkage will increase the survival rate and the prevalence of ARGs (Khachatryan et al., 2004b, 2006b; a, 2008).

These studies suggest that the European Union's recent decision to limit the use of antimicrobial only to cases where they are completely necessary and the susceptibility to the treatment is confirmed is the path to follow. Prophylactic or growth usage of antibiotics should be progressively reduced until its complete disappearance in countries where it is still allowed. Preventive use of antimicrobials should change towards the acceptation of probiotics, prebiotics,

symbiotic organisms and use of organic acids engineered to carry advantageous traits but not the ARGs to compete with the pathogens or resistant species, which will lead to a displacement of the antimicrobial-resistant strains from those niches. In cattle, tetracycline resistance obtained by *tet(A)* or *tet(B)* is very typical. These genes are commonly associated with *tet(R)*, a repressor for their activity. By genetic engineering, it will be possible to add enhancers for *tet(R)* leading to an increase in the fitness cost of bacteria carrying it. This would penalise those bacteria with this gene which will, eventually, be lost. (Shriram et al., 2008). Cases such as mastitis have simpler approaches. This infection can be treated only by rapidly diagnosing the infected animal. Some mastitis are often self-limited, so with enough time, the animal will heal without external help. (Sears and McCarthy, 2003).

11.3. Most relevant ARGs

- Tetracycline: *tet*(B), *tet*(C) *tet*(L), *tet*(M), *tet*(O), *tet*(W).
- Sulphonamide: *sul1*, *sul2*.
- Erythromycin: erm(A), *erm*(B), *erm*(F), *erm*(T), *erm*(X).
- Streptomycin: *str*(A) (Thames et al., 2012; Faldynova et al., 2013; Liu et al., 2019).

12. Approaches to reduce AMR in livestock

12.1. Management

Some diseases are easier to treat at earlier stages, making a quick diagnosis of great importance. There are several biomarkers for diseases such as an increase in liver and kidneys enzymes concentration in blood or protein and cytokines in serum. ELISA or similar laboratory tests can be run to detect these alterations and diagnose the disease before the clinical symptoms appear. Antibiotics would not be needed in that scenario and the animal could be treated with nutritional modulation by supplementing feed with omega-3 fatty acids, phytochemicals and antioxidants. This reduces the incidence of reproductive diseases, ketosis and somatic cells count in milk (Mohammed et al., 2014).

An effort should be made to develop new field diagnosis kits to complement other rapid diagnostic tests like ELISA. It would be of great interest to be able to test an animal for antimicrobial sensibility to prescribe the correct one for that infection. Not using a broad-spectrum antibiotic and going for the most useful one would help to not create new resistances. The downside of this is the increase in the veterinary costs, as a stricter routine and monitoring would be needed. The herd parameters, treatment administered, isolated bacteria and antimicrobial sensitivity should be noted almost daily.

Prebiotics, probiotics and vaccination are some of the approaches to take. Using probiotics that displace the pathogens from their niche could help to prevent diseases. Also, proper vaccination is required as a major biosecurity preventive measure. There are new approaches to take such as using bacteriophages, biological response modifiers or antibacterial peptides. We want to get the

optimal intestinal microbiota to improve the overall animal wellbeing and to reduce the incidence of infectious diseases.
Body physiology is altered under stressful conditions like overcrowding, poor ventilation and temperature control and nutritional deficiency. These conditions result in the secretion of cortisol, a hormone that compromises the immune system, predisposing animals to infectious diseases. Vitamin A, C and E and minerals like selenium, copper and other minerals are used as immunomodulators to reduce the incidence of these opportunistic diseases, decreasing the use of antimicrobials (Ashraf et al., 2019).
Using antimicrobial-free internal teat sealants (ITS) in the dry period have been proved (Burgess and French, 2017) to be as effective as antimicrobial ones at preventing mastitis infections in the following lactation from pathogen present in the environment. The downside is that it brings a new risk of infection if the sealant is not placed under very strict hygienic conditions. The use of ITS could decrease the use of antibiotics by 50% in the dairy industry.
Increasing the biosecurity measures would lead to a lower prevalence of infective diseases. For that, there are some things we can take into consideration like limiting people access to the barn, giving clean clothes different from the ones people are carrying when they need to get closer to the animals, using footbaths and disinfectant or quarantining the new animals before putting them in contact with the existing ones. (Wisconsin Veterinary Diagnostic Laboratory, 2013).

12.2. Nutrition

Regarding nutrition, we can supplement the food with probiotics and prebiotics to avoid the use or the subtherapeutic dose of antibiotics as growth promoters. Some recent studies propose the addition of lactic acid bacteria (LABs) to the diet as they seem to minimise the risk of suffering from mastitis in dairy cattle as they adhere to the mammary gland, occupying the niche that mastitis-causing bacteria take (Rainard and Foucras, 2018).
There are some studies that prove the benefits of using blends of essential oils in milk yield and methane emissions, so further research would be required to understand if they could also boost cow's immune system making them more resistant to bacterial infections (Elcoso et al., 2019).

12.3. Genetics

12.3.1. Microbiota heritability

The heritability of the microbiota is usually calculated using the following equation:

$$h^2 = \frac{\sigma_u^2}{\sigma_u^2 + \sigma_h^2 + \sigma_e^2}$$

Where σ_u^2 is the additive genetic variance of the analysed trait, σ_h^2 is the herd variance and σ_e^2 is the residual variance (Saborío-Montero et al., 2019).

The heritability of the relative abundance of the ruminal microbiota can be estimated using recursive and non-recursive models. For the first one it ranges from 0.08 to 0.48 with a mean of 0.25 and for the latter, from 0.08 to 0.46 with a mean of 0.25. *Prevotella sp., Butyrivibrio sp.* and *Mycoplasma sp.* are the most heritable bacteria (0.34-0.48). The lowest heritability is among *Treponema sp.* and *Fibrobacter sp.* (0.08-0.10) (*figure* 19) (Saborío-Montero et al., 2019).

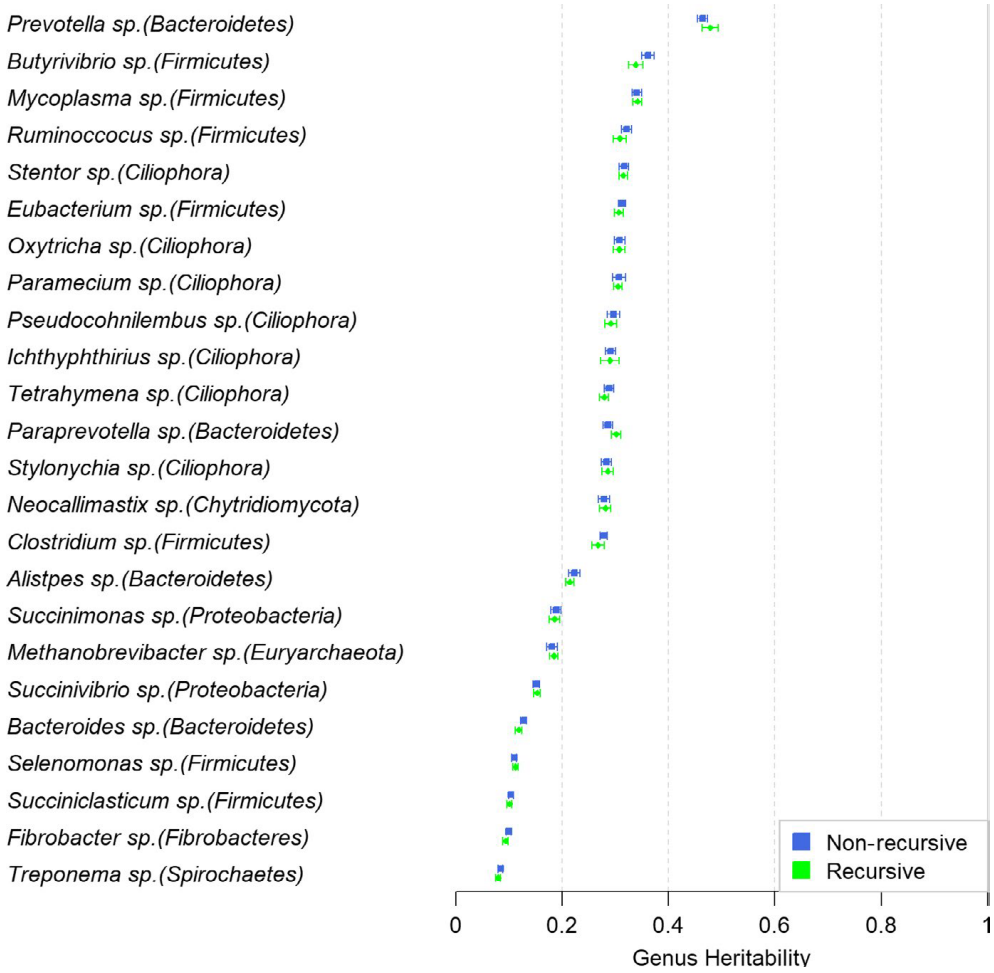

Figure 19 | **Heritability of the microbial relative abundance** | Taken from (Saborío-Montero et al., 2019).

13.3.2. Host effect

Recent studies show that there is a host control over the microbiome. For instance, (Roehe et al., 2016) discovered differences between sire progeny groups on the archaea:bacteria ratio; (Weimer et al., 2010) exchanged ruminal contents and observed that the bacterial composition returned to their original state. (Goodrich et al., 2016) conducted an experiment with human twins and found that the relative abundance of the microbiota has a heritability greater than 0.20. This suggests that the microbiome data could be added to breeding programmes to reduce the methane yield or improve feed efficiency.

(Gonzalez-Recio et al., 2018) studied whether the genotype has any control over the microbiome. For that, several ruminal microbes were selected and analysed for their relative abundance (RA) using diet, age and days in milk (DIM) as covariates. They included two principal components (PC) analysis, the first one to detect stratification at breed level (Brown Swiss vs Holstein) and the second one to get genomic differences among individuals. The former explained 43% of the variance and the latter 10%. The most abundant bacteria were *Bacteroidetes* (48%), *Firmicutes* (32%) and *TM7* (4%). For archaea, the most prevalent clade was *Methanobacteria* and *Methanobrevibacter*.
A host effect could be regulating the composition of the rumen microbiome and thus, some metabolic pathways. We should implement this study to animal breeding programs to obtain animals with a more efficient microbiome which would lead to an improved feed efficiency and a reduction of methane emissions as well as a decrease in the number of pathogens and opportunistic rumen microorganisms.
(Henderson et al., 2015a) classified diets based on forage or concentrate content and gathered the animals according to their feeding. The microbial communities could be separated by host and diet.
Different host fed with different diets revealed that some microorganisms had similar patterns of abundance. Specific correlations should be observed in different diets, hosts and geography within or between archaea, bacteria and protozoa.
It seems that functional redundancy among the microorganisms means that multiple microbial species can fulfil the same function, with different combinations of microbes being co-selected depending on associations between bacteria and archaea.

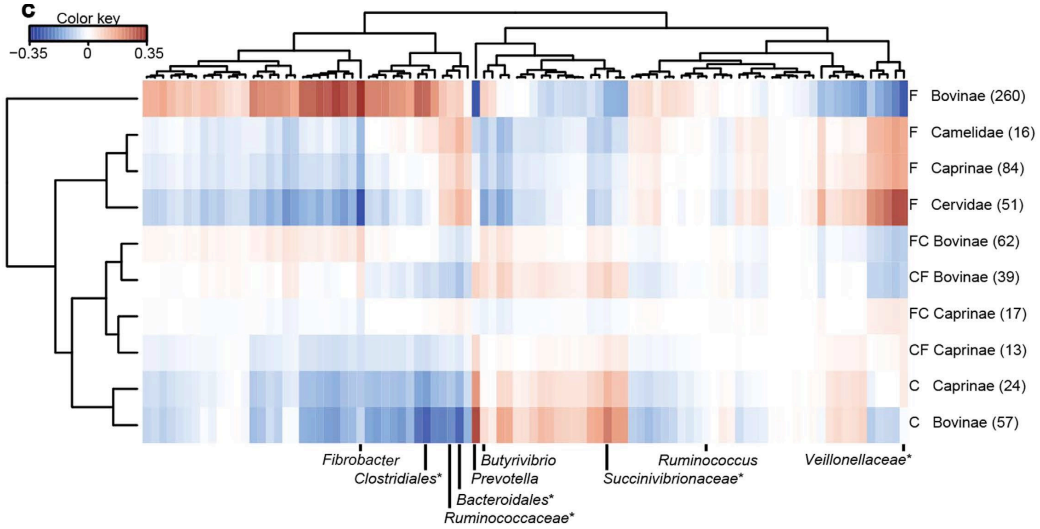

Figure 20 | **Heatmap showing the differential association of bacteria with host and diet.** | Taken from (Henderson et al., 2015a).

(Stewart et al., 2019) investigated the microbiome difference between concentrated-fed and forage-fed cattle. It has been reported that dietary changes can enhance the growth of *Proteobacteria* community (Keto-Timonen et al., 2016)

and regulate stress-response microbial genes (Shin et al., 2015). This is known as "dysbiosis", and it is generally observed after dietary changes or alteration in ruminal volatile fatty acids composition which could be associated with antibiotic treatment, a decrease in the ruminal pH, or an infection with pathogenic bacteria. (Brown et al., 2012). *Proteobacteria*, composed of many pathogenic and opportunistic bacteria such as *Escherichia coli,* is the main phylum found in the rumen along with *Firmicutes*, *Bacteroidetes* and *Actinobacteria*. These bacteria are known to be very opportunistic, which will explain why the growth of *Proteobacteria* accelerates when there is dysbiosis in these animals (Baümler and Sperandio, 2016).
The combined effects of abundance and diversity of pathogens have a huge impact on human health. Diet has a dominant effect on the shedding of the strain O157 of *E. coli*, dietary additives such as monesin and lasalocid included in most feedlots inhibit the growth of Gram-positive bacteria, this alteration gives gram-negative bacteria like *E. coli* a competitive advantage. pH changes caused by dietary changes or dysbiosis could promote the growth of opportunistic bacteria (Callaway et al., 2009).

13.3.3 Breeding programmes and genetic selection

It is of great interest working with a base population and associate its microbiota to their genotypes. Some genes controlling, for instance, the rumen morphology can influence certain bacteria species growth. We could then treat the microbiota as another phenotypical trait and implement it into current breeding programmes thanks to its high heritability. One of the goals would be using haplotypes to predict the animal microbiota.
Selecting for animals with a more favourable microbiota could lead to improve the feed efficiency, reduce methane emission (Saborío-Montero et al., 2019) or even displace pathogens from their niches improving the health of the animal.

14 Brief introduction to metagenomics of AMRs

14.1 What is metagenomics

One of the issues mentioned before was the incapability of growing certain microorganisms in the lab. For that, a new molecular tool to analyse DNA has been developed, called metagenomics. This set of techniques allows us to study the community of microorganisms in an environmental sample, without needing to grow a culture (Ghosh et al., 2019).
The study of the microbiome in livestock species has gained interests in the early twenty-first century from a wide variety of disciplines, including nutrition, physiology and genetics. The early stage of this field and rapid development created the need to standardize protocols, experimental designs and statistical analysis in order to ensure replicability and reproducibility of experiments.

14.2 Planning the experiment and reducing environmental confounding effects

The microbiome profile obtained from diverse samples in the same niche can show a high level of variability. This large variation in the microbiome between individuals can hinder the detection of statistically significant and biologically meaningful differences among experimental groups, especially when the sample size and/or effect are small. In order to conduct a robust microbiome experiment, adequate power and biological replication must be assured and careful attention must be given to numerous confounding factors, such as age, gender, diet, medications, technical or sample processing factors. Main recommendations include exploring the design and results from previous studies in the same type of environment, inclusion of adequate control groups, carrying out pilot studies and collecting as much metadata as possible (recording all possible information about the sample and experimental procedures) and factor these into the subsequent analyses, in order to reduce the influence of confounding factors. Also, repeated sampling of the same individuals over time (longitudinal or time series studies) was suggested to provide a more comprehensive view of the microbial diversity (González-Recio et al., 2019).

14.3 Obtaining and storing samples

Sampling and preservation procedures should be optimised in order to ensure sufficient microbial mass and to minimize contamination. Also, time between sample collection and freezing and the number of freeze–thaw cycles should be reduced as much as possible, as these factors are known to influence bacterial sequence composition (Cuthbertson et al., 2014). Limitations on sample collection may depend on the specific type of sample and its accessibility. While sampling oral, nasal, milk or reproductive tissues can be usually performed with swabs, lavages or direct collection, sampling of ruminal content or ruminal wall requires more complex procedures. Rumen is usually accessed through cannulation or stomach tubing, with both methods resulting in no significant difference in the composition of ruminal microbiome. Stomach tubing is useful to obtain samples from a large number of animals but cannot be used to perform repeated rumen samplings in short periods of time. Faecal samples can also be easily collected by rectal grab with sterile sleeves or with swabs. Gastrointestinal content at other locations can be obtained using endoscope probe or after slaughter. Most widely accepted protocols include snap freezing after sampling, either on dry ice or in liquid nitrogen, followed by long-term storage at –80°C. The effects of short-term storage conditions on diversity and structure of the communities seem to be small (Goodrich et al., 2016). At last, regardless of the procedures chosen, in order to avoid biases in further analyses, collection and preservation methodologies should be standardized for all samples within a given study. Contamination with nonsterile surfaces and any airflow must be avoided, or kept to the minimum (González-Recio et al., 2019).

14.4 DNA extraction

Different methods exist for DNA extraction, with those including mechanical lysis, as bead beating methods being usually preferred over those with chemical lysis and being widely employed for gastrointestinal samples. Nevertheless, the best DNA extraction approach will depend on the underlying microbial composition of a given sample, which is very variable, even within the same type of sample, and thus there is not a single DNA extraction approach that works optimally for all types of sample. Commercial DNA extraction kits are also employed and have been evaluated for microbiome studies in different niches and species. (Vaidya et al., 2018) compared four different protocols for DNA extraction from fibrous and liquid rumen fractions and concluded that every extraction method presented its own strengths and weaknesses in observing specific bacterial families and thus no single extraction method could be proposed or discarded. Although most works performed in cattle were focused on the analysis of rumen microbiome, protocols have also been tested for other sample types such as milk (Lima et al., 2018). It is therefore critical that the DNA extraction method was standardized in a microbiome study.

14.5 DNA sequencing

Analysing the microbial communities of interest in cattle using culture-dependent techniques or other approaches such as denaturing gradient gel electrophoresis (DGGE) or fluorescent in situ hybridization (FISH) is a difficult task.

Illumina®'s platforms use the sequencing by synthesis (SBS) method, meaning that each base is recorded by the device at the same time it is synthesized. Some companies are developing new sequencing technologies to obtain longer sequencing reads. Pacific Biosciences has developed a new device that uses the SMRT (Single-Molecule Real-Time) sequencing to process long reads using the synthesis properties of the DNA polymerases and labelled nucleotides to sequence fragments up to 20 kb long or even larger. Oxford Nanopore Technologies® (ONT) is using protein nanopores to sequence DNA fragments of technically no length limit. Figure 22 shows some of the main particularities of these technologies (González-Recio et al., 2019).

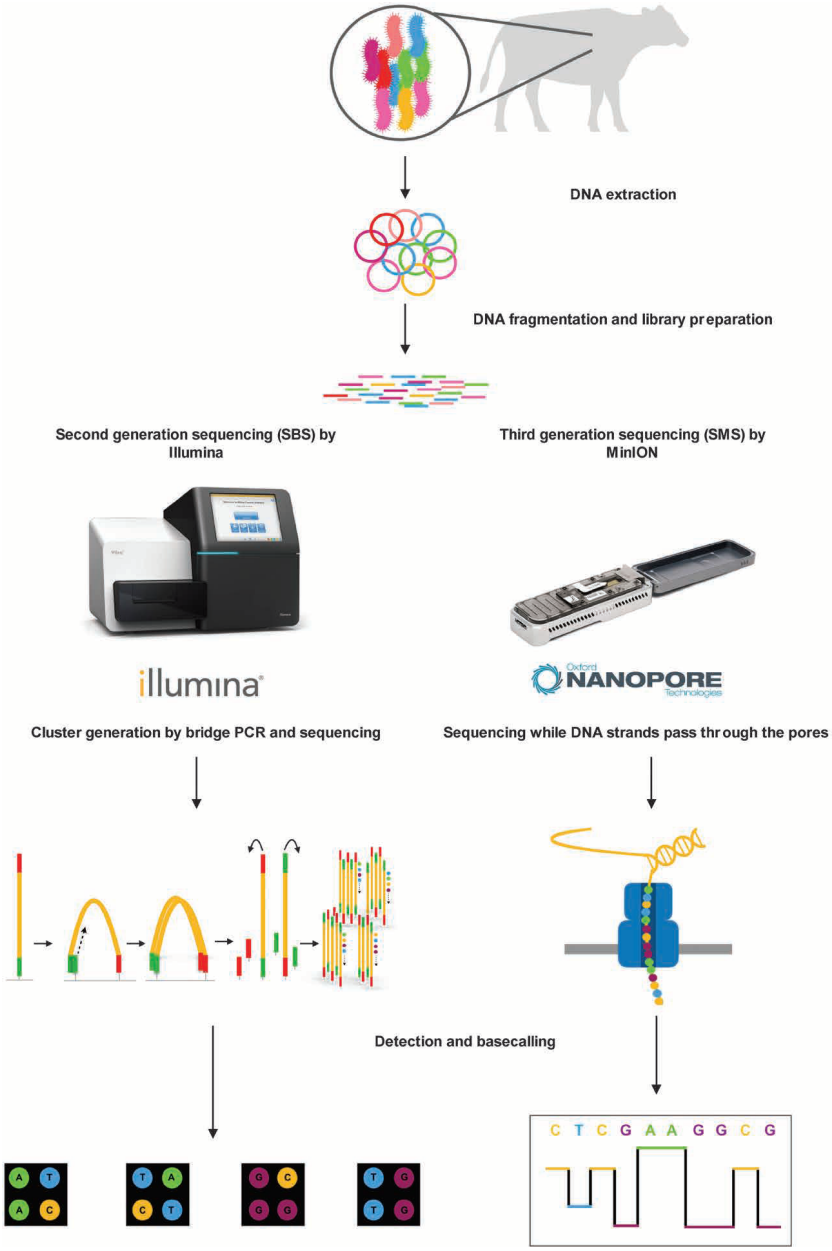

Figure 21 | **Comparison between second and third-generation sequencing platforms** | Taken from (González-Recio et al., 2019).

	Illumina	MinION (Oxford Nanopore)	PacBio RSII
Technology	SBS	SMS	SMS
Read length	150–300 bp[a]	Up to 10 Mb	Up to 200 Kb
Error rate (%)	<0.1	5–15	10–15
Species detection by 16S rRNA gene sequencing	No	Yes[b]	Yes[b]
Weakness	Read length, high initial investment, not suitable for *de novo* assembly	Low accuracy, low throughput	Low accuracy, low throughput, high initial investment
Advantage	High accuracy, high throughput	No capital cost, portability, ultra-long reads, fast results[c]	Long reads, fast results[c]

[a] The throughput and read length depend on the Illumina device (HiSeq or MiSeq).
[b] Long reads allow sequencing of full 16S rRNA gene.
[c] Run times are flexible and are usually less than 1–2 days, depending on the type of experiment.
SBS = sequencing by synthesis, SMS = single-molecule sequencing.

Figure 22 | **Most relevant characteristics of the most used sequencing platforms.** | Taken from (González-Recio et al., 2019).

Next-generation sequencing platforms can be used for taxonomic profiling of different metagenomic samples using two different approaches: targeted amplicon sequencing and whole-genome sequencing (WGS) metagenomics. Targeted sequencing is one of the most used approaches for metagenomic analysis, especially for 16S/18S rRNA gene and internal transcribed spacer (ITS) sequencing. Although targeted sequencing refers to any amplicon of interest, the ribosomal small unit is the most used for taxonomic analysis. It is an essential gene that is present in every living organism. There are two features that make this gene ideal for targeted sequencing: it contains both highly conserved regions that are used to design broad-spectrum primers for the PCR and also variable regions that are specific for each taxonomic group, allowing to discriminate between different microorganisms at low taxonomic levels, even to their genus group. Bacteria and archaea can be classified using their 16S rRNA gene, whilst for eukaryotic organisms their 18S rRNA gene is commonly used as well as the ITS for fungi. This method also relies on large public databases, such as SILVA, Greengenes or the Ribosomal Database Project, which are usually very complete and have thousands of rRNA gene sequences.
Analysing 16S data is usually a straightforward process because there are well-developed pipelines to obtain taxonomic information from the datasets. QIIME and mothur are the two main programs for this task and are usually considered as *gold standards* for 16S rRNA gene analysis. Using targeted amplicon sequencing has become a very useful tool for taxonomical analysis of environmental samples and has led to an increase in the diversity that can be found in a concrete environment compared to the diversity found when using classical techniques. However, taxonomic classification using this method does not allow species detection and lacks functional information. Besides, taxa are assigned analysing only one region of the complete bacterial genome and the primers used for the PCR could lead to under- or over-representation of some taxa. Shotgun metagenomics is gaining prominence as a powerful tool to define

microbial populations from all types of environments. With the advent of more affordable prices for WGS, more researchers are now using WGS metagenomics to obtain functional information about species and for *de novo* assembly of microbial genomes. This strategy is PCR-free, generating millions of reads that should cover the entire genome or genomes present in the sample, providing information about taxonomy and functionality. Functional information is usually useful to describe new environments and to associate them with the microbial profiles found in a sample, as well as detecting relevant biological processes, enzymes and proteins involved (González-Recio et al., 2019).

14.6 Data analysis of AMR

Once the data has been obtained, we need to find the ARGs present in our samples. For that, there are several tools that we can use to map our sequences against antimicrobial resistance genes databases.

- Resfinder is a web server that uses BLAST to identify the ARGs present in our samples (Zankari et al., 2012).
- The Comprehensive Antibiotic Resistance Database (CARD) provides reference DNA and protein sequences, detection models and bioinformatics tools on the molecular basis of bacterial antimicrobial resistance (Alcock et al., 2019).
- Resfams can be used to search for conserved structural domains of AMR in protein sequences (Sabino et al., 2019).
- ARG-ANNOT detects existing and putative antibiotic resistance genes in bacterial genomes (Gupta et al., 2014).
- NanoARG analyses data produced by nanopore sequencing technology, profiling ARGs, MRGs, MGEs and putative pathogens (Arango-Argoty et al., 2019).

15 Materials and methods

This study was conducted in the Department of Animal Breeding of the National Institute for Agricultural and Food Research and Technology (INIA), Madrid, Spain.

15.1 Obtaining the data

The data used to carry out this master's thesis comes from the project "METALGEN" which aims to improve feed efficiency and mitigate the emission of greenhouse gases in dairy cattle.

It is a multidisciplinary project coordinated by INIA, NEIKER and CONAFE that aims measuring methane emissions in farms in Spain and investigate the associations between methane, the microbiota, diet and host genetics. The final objective is to elaborate new nourishments and incorporate feed efficiency and lower emissions in the national breeding program in dairy cattle, leading to a decreased use of natural resources and reducing the carbon footprint in the cattle industry.

During sampling collection, cows are placed in individual stalls and a tube is introduced down their oesophagus to their rumen. Around 100 ml are then pumped out and stored in a container. The solid fraction is filtered using four layers of cheesecloth and frozen in liquid nitrogen (N_2) immediately. Frozen samples are transported to the laboratory in liquid N_2 and stored at -80ºC until analysed. Data comes from 14 commercial farms in Cantabria, País Vasco, Navarra y Cataluña. Rumen samples were extracted from 472 Friesian cows, but after quality control a total of 439 were analysed using the CARD pipeline.

Methane emissions were measured using an infrared detector (The Guardian© NG infrared gas monitor from Edinburgh Sensors) placed inside the trough where cattle feed when being milked by the robot. Each cow's methane concentration in breath in measured individually during milking for 14 days. There was availability of all traits related to milk yield and composition for each one of them thanks to the milk control performed during those 2 weeks.

Cows were genotyped using the EURO12K SNP chip from Illumina and imputed to 54,609 SNPs (Bovine 50k SNP chip, Illumina) using BEAGLE software and the Spanish reference population provided by CONAFE (Spanish Friesian Associations Confederation).

Samples were thawed and homogenized using a blender before being analysed using the commercial kit "DNeasy PowerSoil" (Qiagen). The concentration and purity of each sample was estimated using a NanoDrop UV/Vis Spectrophotometer (NanoDrop Technologies Inc.). The sequencing was performed using Nanopore Technology and the MinION sequencer, following the protocol from Oxford Nanopore (Oxford, UK) to prepare the sample multiplexing, barcoding and the library.

15.2 Bioinformatic analyses

The sequences were analysed using the SQMreads tool from SqueezeMeta (Tamames and Puente-Sánchez, 2019), a pipeline for metagenomics. It aligns each read to a gene reference database and provides the number of copies of each

gene present in the sample. We implemented a custom database related to antimicrobial resistances, the Comprehensive Antibiotic Resistance Database (McArthur et al., 2013) for genes to be assigned to the CARD ontology for taxonomy and function annotation. This pipeline was implemented in the CESGA super-computing centre.
The output was a tsv file with 989 rows, 988 for each gene and 1 for the unclassified reads, which would be the non-ARGs and 439 columns, one for each animal.
Each column represents an animal from one of the 14 farms, except the first one called "X" that contains the accession number of the antimicrobial resistance gene. For instance, ARO:3000535 represents the gene *macB*.

X	AGE_001	AGE_002	AGE_003	AGE_004	AGE_005	AGE_006
ARO:3004507	0	0	0	0	0	0
ARO:3004509	0	0	0	0	0	0
ARO:3004510	0	0	0	0	0	0
ARO:3004511	0	0	0	0	0	0
ARO:3004513	0	0	0	0	0	0
ARO:3004515	0	0	0	0	0	0
ARO:3004516	1	0	0	0	1	1
ARO:3004517	0	0	0	1	2	0
ARO:3004541	0	0	0	0	0	0
ARO:3004542	0	0	0	0	0	0
ARO:3004544	0	0	0	0	0	0
Unclassified	102017	210002	87146	131317	97838	127959

Figure 23 | **Example of how the reads are visualised.** Each row represents an ARG and each column, an animal.

15.3 Treating our data as compositional data

The reads fall into the category of compositional data (CoDa), as they are discrete vectors representing the numbers of outcomes falling into any several mutually exclusive categories. This count data sets can contain zero values which are often the result of insufficiently large samples.
The main issue with CoDa is the difficulty to differentiate between real and false zeros. For that, generalised Bayesian-multiplicative replacement was used (Martín-Fernández et al., 2015). This technique is appropriated when the total sum of a vector is uninformative, as happens in this case when the interest lays in the relative abundance of each gene. With $c_i = (c_{i_1}, \dots, c_{i_D})$ as a compositional vector of counts, gene reads in this case, with some zeros and $n_i = \sum_i c_{i_j}$ the BM replacement replaces $x_i = \frac{c_i}{n_i}$ is replaced by the vector $r_i = (r_{i_1}, \dots, r_{i_D})$.
A zero is replaced by its posterior Bayesian estimate $E[\pi_i|c] = \frac{c_i + s \cdot t_i}{n+s}, t_j = \frac{1}{D}$ using the following formula:

$$r_{ij} = \begin{cases} t_{ij} \cdot \frac{s_i}{n_i + s_i}, if\ x_{ij} = 0, \\ x_{ij} \cdot \left(1 - \sum_{k|x_i k=0} t_{ik} \cdot \frac{s_i}{n_i + s_i}\right), if\ x_{ij} > 0, \end{cases}$$

Being t_{ij} related to the prior and s_i to its strength. The parameters may vary along the samples according to the information of the trials. The advantage of this technique is the preservation of the ratios between parts and the sum of the vector:

$$\frac{r_{ij}}{r_{ik}} = \frac{x_{ij}}{x_{ik}}; \sum_{j=1}^{D} r_{ij} = 1$$

For this, genes with a total sum of reads smaller than 3 are removed from the data set. The remaining are run through the Geometric Bayesian multiplicative (GBM) method from the cmultRepl function of the zCompositions package in R (Palarea-Albaladejo and Martin-Fernandez, 2015).

15.4 Who's there? Microbiota composition.

For this process, the pipeline of work has been similar to the one for the ARGs. The negative value in the unclassified row is removed from the dataset and the "X" column containing the names of the microbes is copied to the row names so this column can be removed, and the zeros imputed. Microbes with fewer than 3 reads are removed from the data. The microbiota data given in reads has been imputed using the cmultRepl function from the zCompositions package and the RA of each superkingdom, phylum and class, genera and species was calculated. The results are represented in a stack plot.

The taxonomy file does not distinguish between superkindoms, so the first step is to create subsets of data for each one of them: archaea, bacteria, eucharyota and viruses. Names need to be modified, special characters such as "/" or "()" need to be removed as it can cause misunderstandings with the functions.

```
RA_phylum_eukaryota$aro <- gsub("k_Eukaryota;p_","",RA_phylum_eukaryota$aro)
RA_phylum_eukaryota$aro <- gsub("\\(no phylum in NCBI\\)","no
NCBI",RA_phylum_eukaryota$aro)
```

Figure 24 | **An example of how the names need to be trimmed.**

15.5 Calculating the relative abundance of antimicrobial resistance genes

Data were grouped into herds and the relative abundance of each gene was calculated. In a first approach, the 20 most prevalent genes of each farm are determined, resulting in a list of 25 genes. Later, genes with a prevalence greater than 0.005% are studied individually, resulting in a list of 69 genes for which AMR gene family, class, resistance mechanism and resistomes were obtained.

As shown in the image below, first we select with which herd we want to work and calculate the mean of the reads of each gene within the herd to be able to obtain the relative abundance of them. We arrange the values in a descendent order and keep the 20 most prevalent genes. The most prevalent one is not selected as it represents the Unclassified column, that contains no ARGs.

```
RA20prevARGs <- function(herd,df){
  rebano <- (df %>%  dplyr::select(starts_with(herd)))
  herdname <- c(rep(herd,nrow(df)))
  herd.mean <- apply(rebano,1,FUN=mean) ##El 1 significa fila el 2 columna.
  herd.RA <- RelativeAbundance(herd.mean)
  aro <- colnames(carddataImp)
  herdandgene <- data.frame(aro,herd.RA)
  RAarranged <- arrange(herdandgene, desc(herdandgene$herd.RA))
  RAfinal <- data.frame(RAarranged,herdname)
  prevgenes <- RAfinal[2:21,]
  return(prevgenes)
}
```

Figure 25 | **Function to calculate the 20 most prevalent ARGs by herd.** Firstly, all the data belonging to a herd is extracted from the data frame. A mean is applied to the number of reads for each ARG and the relative abundance of each ARG is calculated. Finally, the 20 most prevalent ARGs of that herd are kept and returned, removing the first row which contains the unclassified reads.

Genes with a prevalence greater than 0.005% are plotted wrapping the results by herd, obtaining 14 plots to compare whether the most prevalent genes are the same ones in all the farms or there are differences between them. For this a data frame with the RA of the ARGs of each herd is created. This table contains the information needed to plot the results: the accession number of the gene, the name of the gene merged by the card ontology which had been previously modified to follow the same pattern (ARO:XXX) as the one in our output file, the RA of the ARGs and the herd to which it belongs.

aro	Name	herd.RA	herdname
ARO:3000191	tetQ	0.028877786	MEND
ARO:3000191	tetQ	0.015208875	MIREN
ARO:3000191	tetQ	0.017860874	SH
ARO:3000191	tetQ	0.061185674	MASG
ARO:3000191	tetQ	0.018258795	AREN
ARO:3000191	tetQ	0.027919061	AR_
ARO:3000191	tetQ	0.025534620	AMET

Figure 26 | **Representation of the RA by herd dataframe.** Each gene and its unique aro are shown besides its RA and the herd to which the gene belongs.

This data is then plotted using the ggplot2 package (Wickham et al., 2020) wrapping the data by herd.

```
ggplotly(ggplot( data = ARGsRA20prevs, aes(x = Name, y = herd.RA, fill = Name)) +
  scale_fill_manual(values = c25) +
  geom_bar(stat = "identity") +
  facet_wrap( ~ herdname) +
  ggtitle("Relative abundance by herd") +
  theme(plot.title = element_text(hjust = 0.5),
        legend.text = element_text(size = 5),
        axis.text.x=element_blank(),
        axis.ticks.x=element_blank()) +
  ylab("Relative abundance") +
  xlab("") +
  guides(fill=guide_legend(title="ARGs")) )
```

Figure 27 | **Code used to print the plots.** Genes are represented in the x axis, and their RA in the y axis. Colours are assigned to each gene manually and all the plots are wrapped by the herds.

The integration of CARD is necessary as it will allow the association of each gene with its AMR families and being possible to calculate the percentage of each family and the most common antimicrobials used in the dairy cattle industry associated to them.
The .obo file available to download the database information contains the ID of the gene or the resistance mechanism, the name of the same and its definition as shown below:

```
[Term]
id: ARO:3000535
name: macB
namespace: antibiotic_resistance
def: "MacB is an ATP-binding cassette (ABC) transporter that exports macrolides with 14- or 15- membered lactones.
is_a: ARO:3000748 ! subunit of efflux pump conferring antibiotic resistance
relationship: part_of ARO:3000545 ! MacAB-TolC
synonym: "pvdT" EXACT []
xref: PDB:3FTJ
```

Figure 28 | **CARD ontology example.** The id is the accession number or "aro" of the gene. "is_a" contains the family of resistance and "def" shows a brief definition of how the resistance works.

This file is integrated into our script as a list using the get_ontology function from the ontologyIndex package for R (Greene et al., 2017).

15.6 Category and family assignation

The 69 most abundant genes of each herd are isolated as explained above, and then their antimicrobial resistance family and the mechanism of resistance that such family confers are annotated. The RA of each family is calculated and plotted.
Antibiotics are classified in categories according to when their use is allowed as explained in the introduction. The antibiotic to which each gene confers resistances is not included in the ontology file. The 69 most prevalent genes in each herd were isolated and studied individually. The class of antibiotic(s) to which it confers resistance can be found in the CARD website. Most of the genes confers resistance to more than one antibiotic, as the resistance lies in the mechanism and not the ATB itself. Even if two ATBs are used to treat diseases completely different, both can be resisted by the same mechanism as the

resistance is based on where the ATB acts (if it inhibits a transport chains or binds to a bacterial receptor). Once the gene is associated to the class, we assign the category of the antibiotic to that class (A, B, C or D). RA of each class is calculated, and the categories are plotted.

Gene	Category	Class
adeJ	A	Carbapenems
adeJ	A	Rifamycin
adeR	A	Glycylcycline
Bifidobacterium adolescentis rpoB mutants conferring...	A	Rifamycin
carA	A	Oxazolidinone

Figure 29 | **Classification of genes by the category of ATB to which they confer resistance.**

15.7 Bacteriophages correlation

To calculate the correlation of the bacteriophages with the ARGs it is necessary to work with the phylogenetic outputs of SQM reads. The file is opened in R and imputed the same way as the ARGs file. Once this is performed, RAs are calculated, and bacteriophages are filtered and merged with the information related to the RA of the most prevalent ARGs.

Around 95% of the bacteriophages belong to the "caudovirales" order, dsDNA tailed viruses that infect bacteria binding to receptors in their membranes (Xu et al., 2004). This is the reason because this order has been selected along with the explicit phages to be grouped into a "total RA of phages" category created to simplify the calculation of the correlation with the ARGs.

Then, the function "cor" (R Core Team, 2020) is used to create a correlation matrix of the bacteriophages with the ARGs, resulting in a 26x26 matrix which results are plotted using the corrplot function (Wei and Simko, 2017) using the "color" method.

15.8 Calculating heritabilities and correlations

Heritabilities of the ARGs and their correlation with productive traits (milk, protein and fat yields as well as methane emissions) were calculated using a modified version of the software Threshold Model (Legarra et al., 2011) to include the genomic relationship matrix instead of the pedigree numerator matrix. For this, cows needed to have been genotyped, analysed by SQMreads and have milk yield records. A total of 416 out of the 472 cows meet these requirements. Phenotypic correlation between bacteriophages present in the ruminal microbiota and ARGs were also calculated using a correlation matrix.

The correlation of the 25 most prevalent genes has been calculated with milk, protein and fat yield and methane emissions, for that, a file for each gene with each trait need to be created (*figure* 23). In the files, each row represents an

animal. The first column is the covariable (mean), the second one the number of lactation of the animal (first or second), the third one is the herd to which the cow belongs (from 1 to 14), the forth one is the days in milk in which the cow is (1 to fewer than 70 days, 2 from 70 to 150 and 3 for more than 150), the fifth one contains the random effect, which is the number of animals the sixth column contains the values of the trait of interest and the seventh the RA of the ARG. As RAs of ARGs values are very small compared to those of the CH4 emissions, the CH4 values were divided by 100 to simplify the calculations.

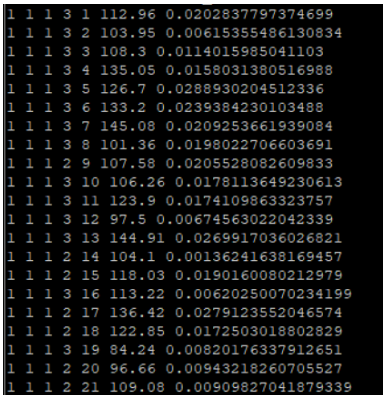

Figure 30 | **Example of how the TM files need to look like.**

TM computes posterior distributions for variance components and relevant ratios (heritabilities, correlations) (Legarra et al., 2011). It uses Markov chain Monte Carlo (MCMC) and Gibbs sampling to always work under the same core and fixed effects and variance components are calculated under flat priors. The idea of using thresholds is that over a given one, there will be a given phenotype. At each iteration, the program generates a liability below or over the threshold such as the value is 0 or 1.
In the case of this study, the number of iterations for each pair of gene-trait is set to 300,000, with a burn-in of 100,000 and a thin interval of 10 as shown in figure 31. This means that the program will discard the first 100,000 iterations and take samples each 10 iterations from the 100,001 to the last one.

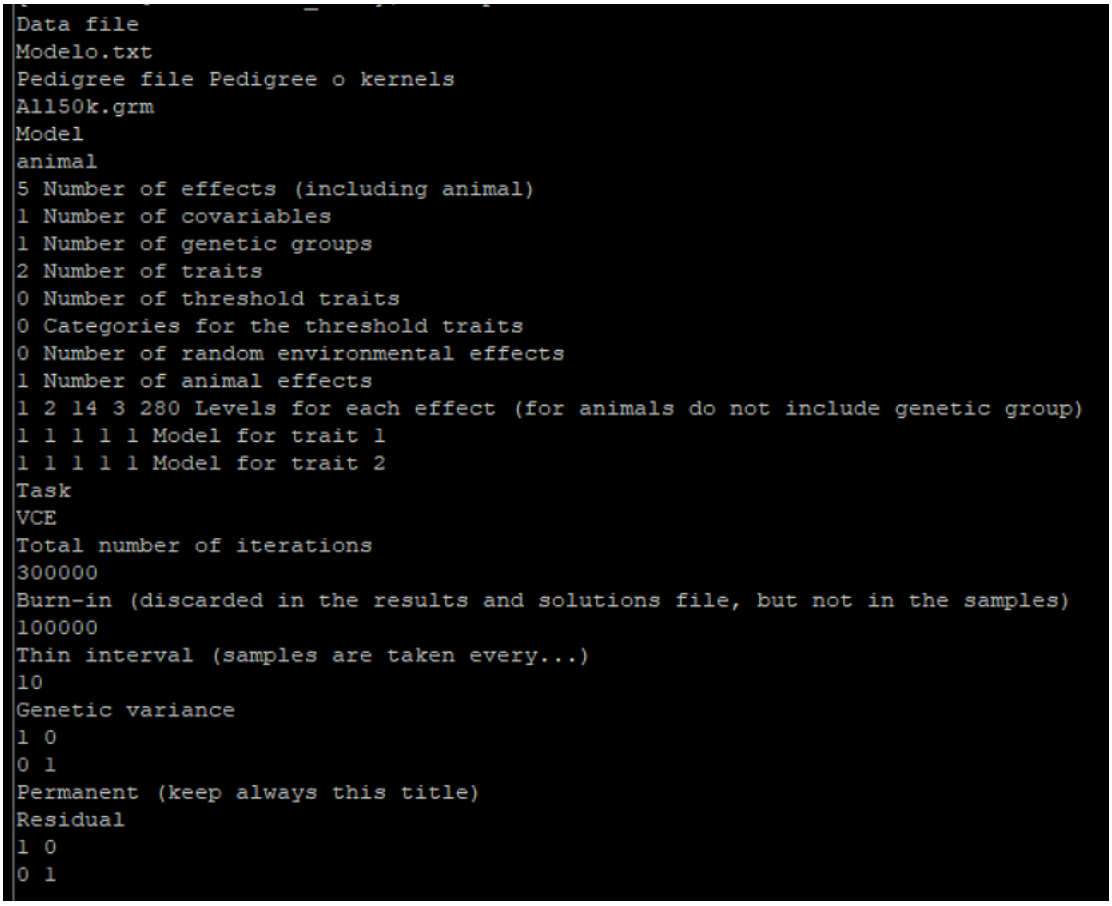

Figure 31 | **Example of how the parameter file needs to be prepared.**

The model used is recursive, meaning that, in this case, the RA of the ARGs will affect the CH_4 emissions either positively or negatively but this increase on CH_4 emissions will not affect the RA of the ARGs. This is what lambda represents in the first equation, the recursive effect of the antimicrobial resistance genes over the emitted methane (Saborío-Montero et al., 2019).

$$\boldsymbol{y_{CH4_{ijklm}} = \mu + LC_j + Herd_k + DIM_l + \lambda(RA_{ARG_m}) + e_{ijklm}}$$
$$\boldsymbol{y_{RA_{ARGs_{ijkl}}} = \mu + LC_j + Herd_k + DIM_l + e_{ijkl}}$$

where: LC, lactation; DIM, days in milk; RA ARG, relative abundance of the antimicrobial resistance genes; i, i-th animal; j, j-th lactation, k, k-th herd; l, l-th day in milk and m, m-th relative abundance of antimicrobial resistance genes.
The error of the heritabilities is calculated using a confidence interval of a 95% High Posterior Density (HPD) for each one of them. For that, the following formula is used: quantile(h2,probs = c(0.05,0.95),na.rm=T). We are producing sample quantiles corresponding to the probabilities (0.05, 0.95).
The correlation of each ARG and its standard deviation are calculated using the TM software output. These correlations need to be corrected by lambda, which measures the strength of the correlation of each gene with the productive trait. For this, the following formula was used:

$$Cor = \frac{covar + \lambda * va2}{\sqrt{va2 * (va1 + 2\lambda * covar + \lambda^2 * va2)}}$$

Where covar is the covariance the trait and the gene, va1 is the variance of the trait and va2 is the variance of the ARG (Gianola and Sorensen, 2004).

The heritabilities are calculated using the TM software and represented using the viz_forest function (Kossmeier et al., 2020). This allows us to create a thick forest analysing our heritabilities and the high posterior density (HPD) to represent the values and their confidence intervals (Lewis and Clarke, 2001). The heritabilities are also grouped by the mechanism of resistance that the ARG confers. The heritability is calculated using the following formula:

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_e^2}$$

Where σ_a^2 is the additive variance and σ_e^2 is the variance of the error.

16 Results

16.1 Data obtained

A total of 988 genes conferring resistance to antimicrobials in the ruminal ecosystem were identified. Sixty-nine of them, which are strongly represented in all herds, have a relative abundance higher than 0.005%, making up what we can refer to as the core resistome.
We have then 997 ARGs with 957801 reads and 96848079 reads for non-ARGs genes, ARGs making up 0.9889726% of the total.

16.2 Treatment of zeroes

To impute those values, the Bayesian-multiplicative replacement (GBM) corrected 31849 values of the 324996 in our dataset. Meaning that 9.8% of the total values were corrected.

16.3 Who's there? Microbiota composition

This section shows the microbial composition found in the samples used in the study.

16.3.1 Superkingdoms

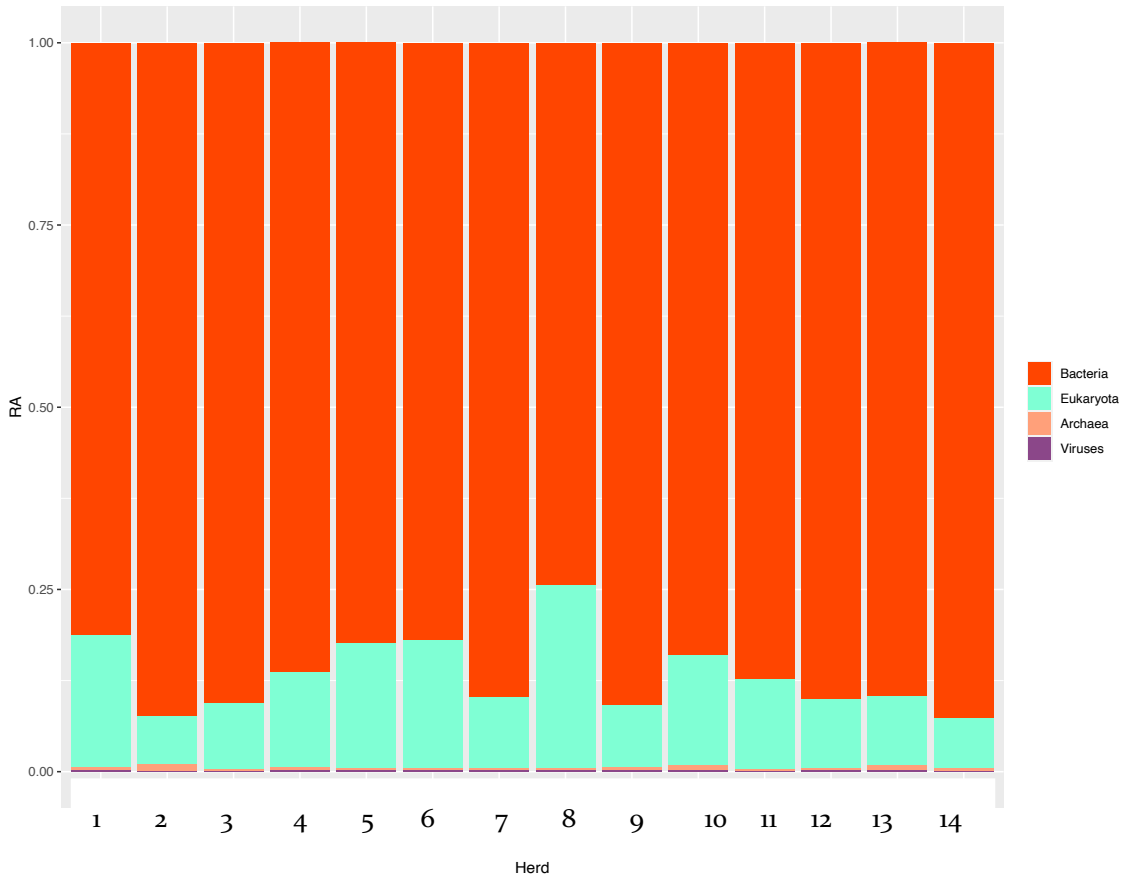

Figure 32 | **RA of the microbiota superkingdoms.** The stacked bars represent the RA of a given microorganism in each herd. A colour can only be associated with a single microorganisms, which is shown in at the right of the image.

Figure 32 shows that most of the rumen microbiota is made up of bacteria and eukaryote as both groups are responsible for breaking down the fibre that the animal's enzyme cannot digest. Other studies suggested that the RA ranges between 0.3 to 3% (Janssen and Kirs, 2008) and our results (0.83-0.22%) fit into these values. (Janssen and Kirs, 2008) also proposed that only strict anaerobic methanogenic archaea are found in the rumen. The RA of viruses was around 0.1 to 0.2%. This is a good health indicator as viruses only have pathogenic roles.
Most of the farms have similar bacteria-eukaryote ratio. The variation of the RA of eukaryote was most likely explained by the effect of feeding in the different farms.

16.3.2 Archaea

16.3.2.1 Phylum

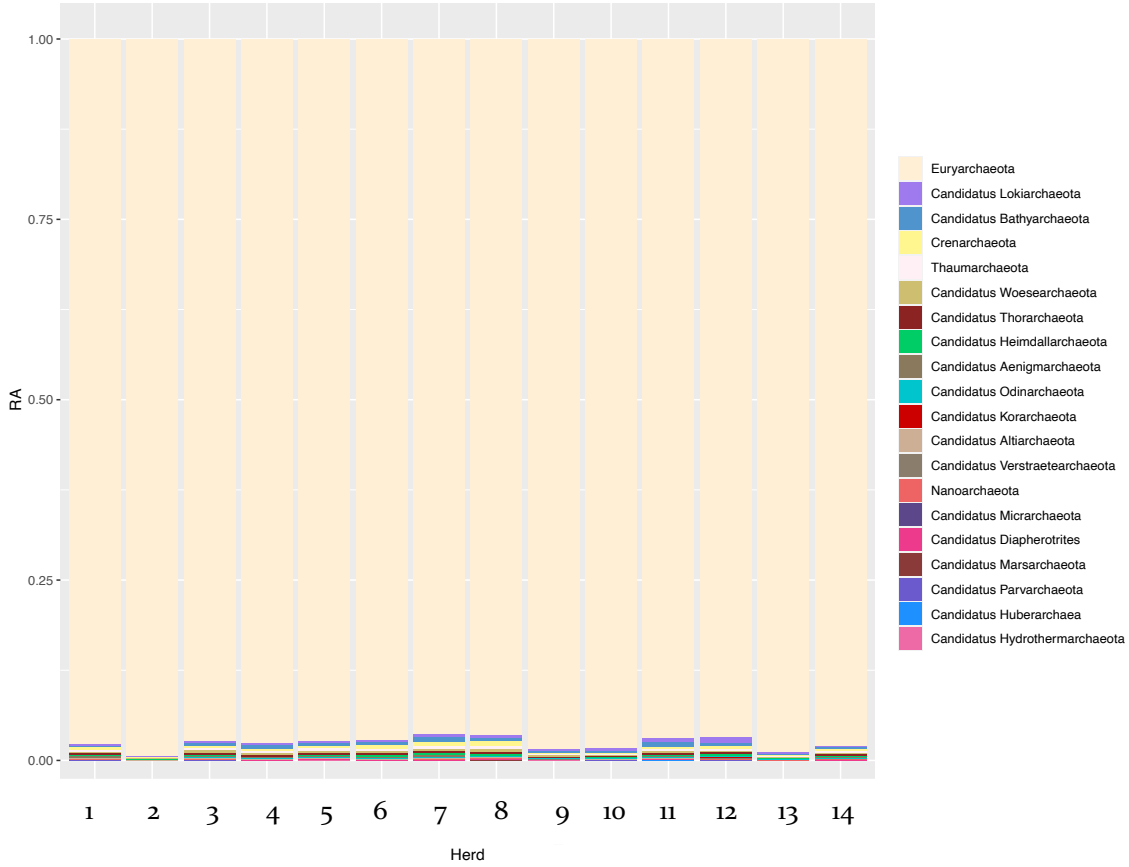

Figure 33 | **RA of the archaea phylum.**

As shown in the figure 33, *Euryarchaeota* was the most abundant phylum of archaea in our samples. Most classes belonging to *Euryarchaeota* (*Methanomicrobia* and *Methanobacteria*) are involved in methane emission (Villa Gomez et al., 2019). *Thermoplasmata* are a class of archaea distinguished for being acidophiles and most of them thermophilic also responsible for methane emission as they have been described as methylotrophic methanogenic microorganisms (Poulsen et al., 2013). These classes are represented in figure 34.

16.3.2.2 Class

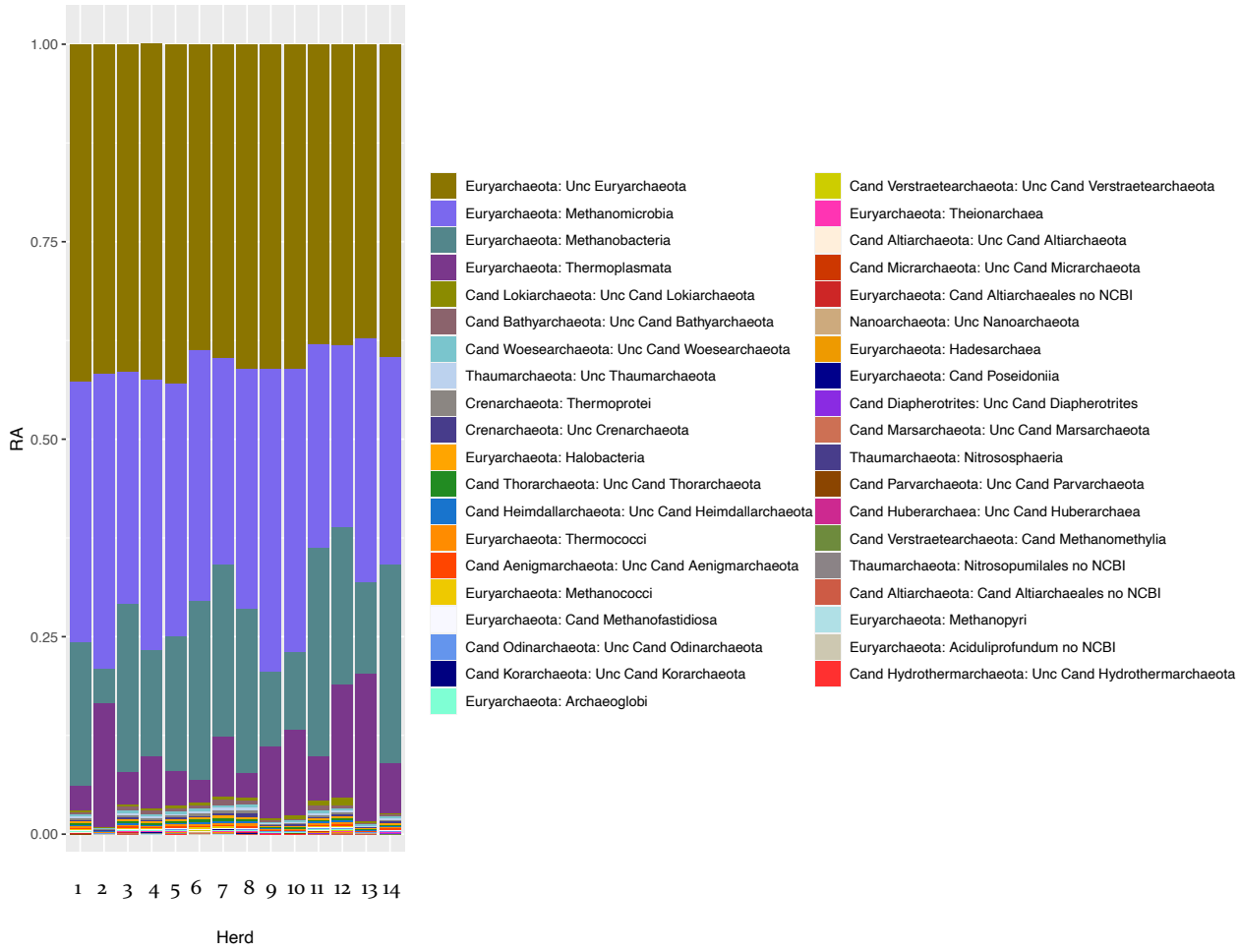

Figure 34 | **RA of the archaea classes.**

16.3.3 Bacteria

16.3.3.1 Phylum

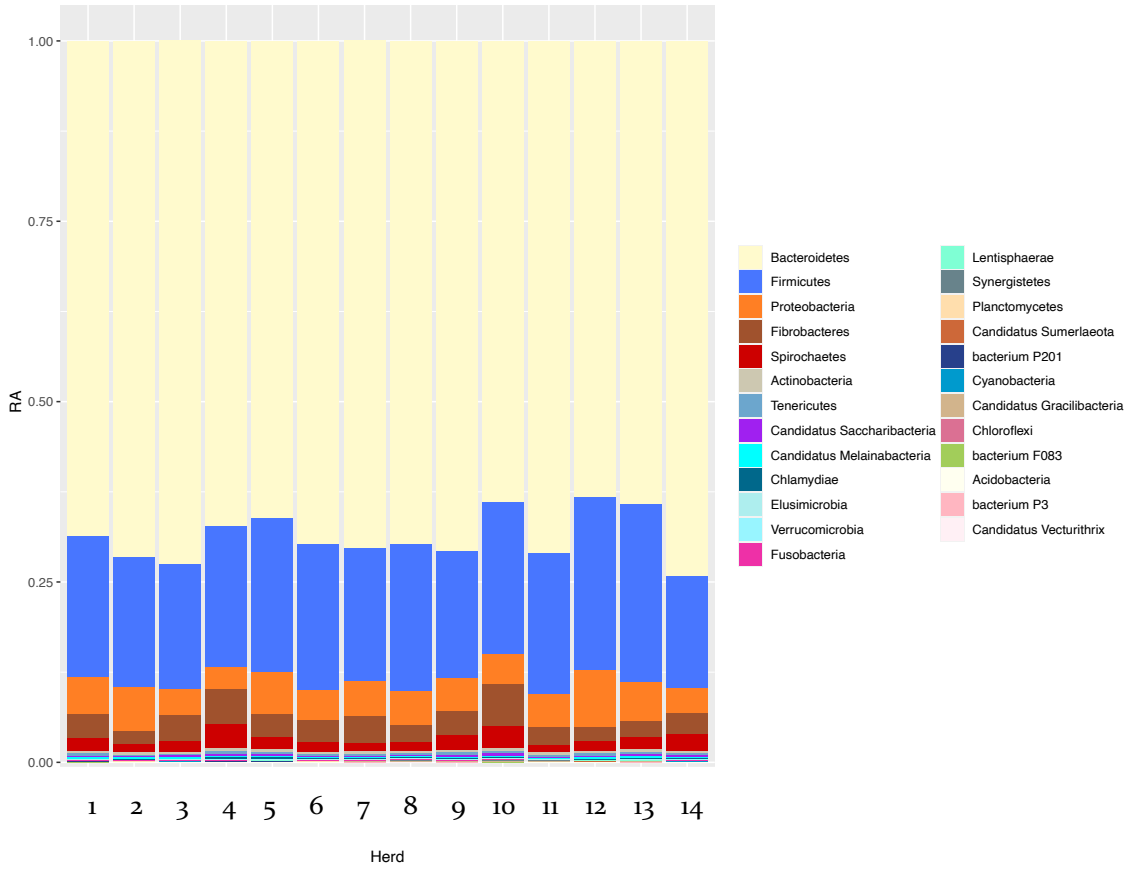

Figure 35 | **RA of the bacteria phylum.**

Figure 35 shows that the most prevalent bacterial phylum found are *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Fibrobacteres*. *Bacteroidetes* and *Firmicutes* were the most abundant phyla in the rumen. These bacteria carry a large amount of glycoside hydrolases (GHs) and polysaccharide lyases (PLs) genes in their genomes that are necessary for fibre digestion. Ruminants rely on these enzymes to be capable of digesting and breaking down fibre. *Bacteroidetes* have a larger amount of copies of these genes than *Firmicutes*, so an increase in the *Bacteroidetes-Firmicutes* ratio may not be desired.

As explained in the introduction, *Proteobacteria* are opportunistic bacteria that can grow when the animal is in dysbiosis after dietary changes. It is important to keep the *Proteobacteria*/(*Bacteroidetes*+*Firmicutes*) lower than 0.19 as a higher value could promote the growth of pathogenic bacteria (Auffret et al., 2017).

Bacteroidetes were more abundant in first lactation cows as they are involved in the majority of their metabolic functions. The RA of *Firmicutes* and *Proteobacteria* increase incrementally in further lactations (Khafipour et al., 2016; Pitta et al., 2016b).

16.3.3.2 Class

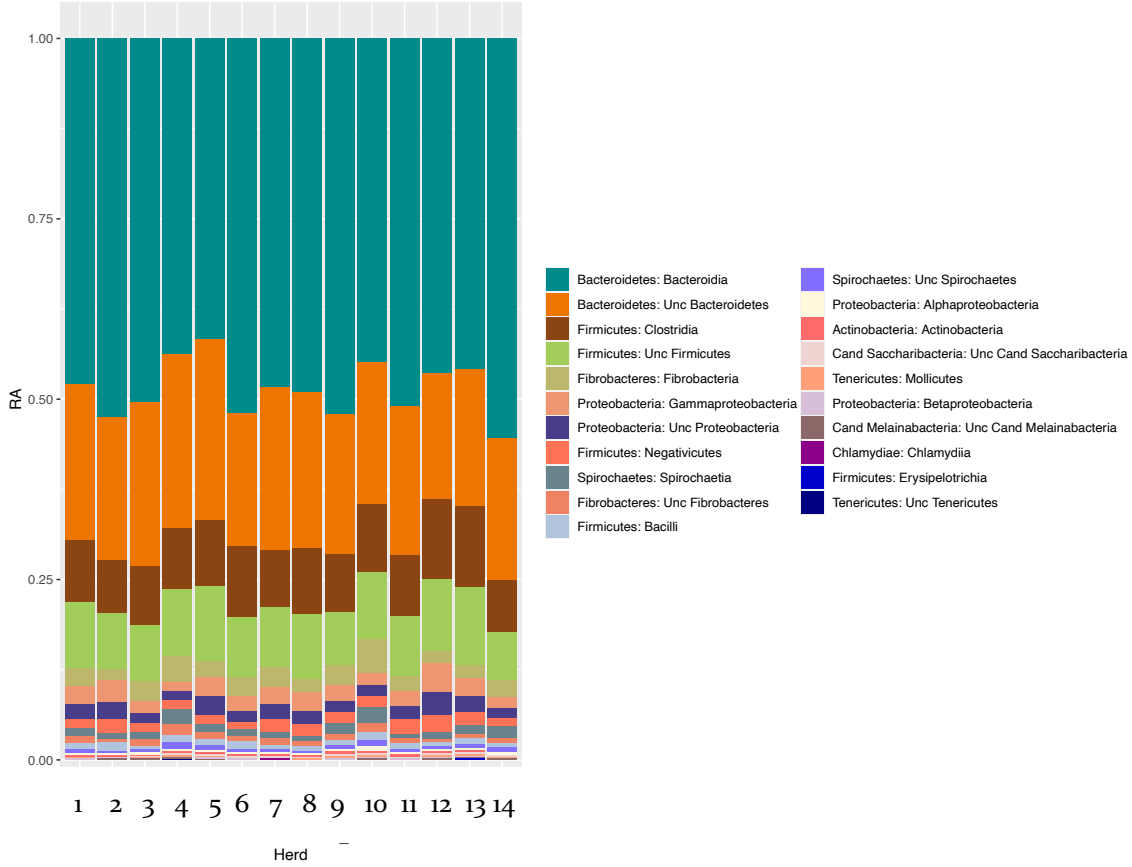

Figure 36 | **RA of the bacteria classes.**

At the class level we observed a large RA of *Bacteroidia* and Clostridia which play a role in plant fibre degradation. (Cunha et al., 2011).

16.3.3.3 Species

In the figure 37 we can observe that *Prevotella*, *Firmicutes* and *Bacteroidetes* are the most abundant phyla as they are involved in the metabolism of proteins and peptides and milk yield (González-Recio et al., 2019). *Treponema bryantii* is also observed in every herd as it interacts with cellulolytic bacteria (Stanton and Canale-Parola, 1980).

The problem lies in the presence of *Staphylococcus aureus* in all the samples, a commensalistic bateria with some strains responsible for the most common of contagious mastitis and it is strongly connected with resistance to methicillin. Although the presence of *S. aureus* is common in all the living organisms and only certain strains are pathogenics, nonetheless we need to be aware of its presence.

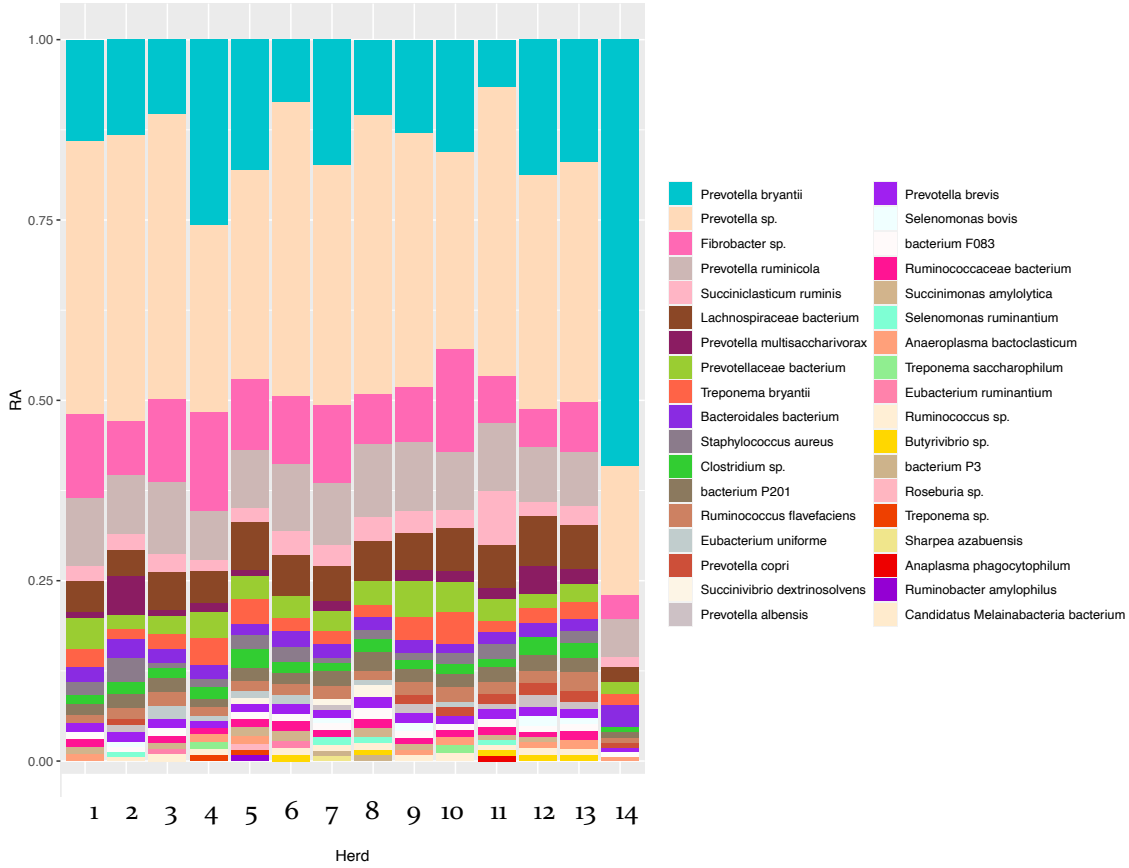

Figure 37 | **RA of the bacteria species.**

16.3.4 Viruses

16.3.4.1 Phylum

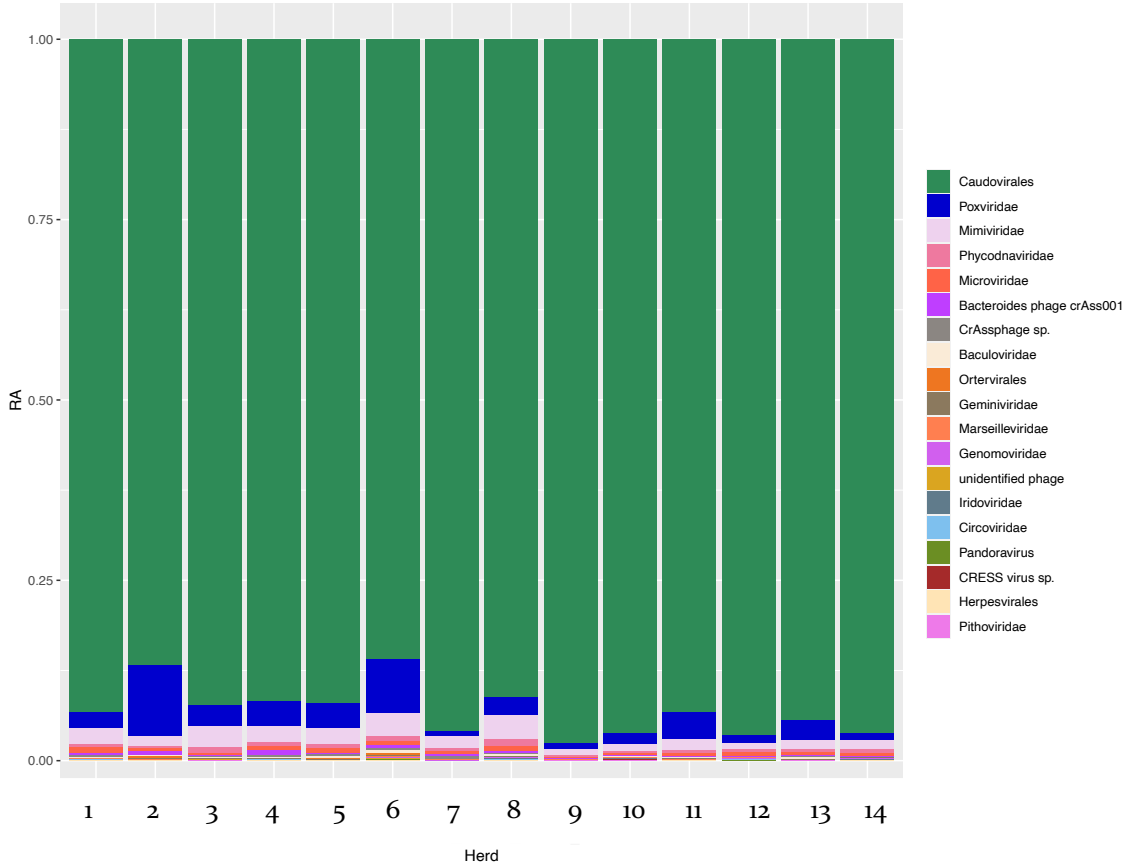

Figure 38 | **RA of the virus phylum.**

Horizontal gene transfer by viruses and phage has played a major role in the evolution of prokaryotes (Koonin and Wolf, 2008). This transfer is mediated by plasmids, transposons and viruses; mechanisms known as the mobilome.
Classification of viruses is highly complicated. The information available in the National Centre for Biotechnology Information (NCBI) is not yet fully comprehensive, and phylum, order and family are not highly accurate. Nonetheless, figure 38 shows the resulted classification in the virus kingdom. *Caudovirales*, from phylum *Uroviricota* (Adams et al., 2016), were the most abundant in this study. These viruses are an order of dsDNA tailed bacteriophages (Christie and Dokland, 2012). *Caudovirales* cleave part of the chromosomal DNA of the infected bacterium when they replicate their own genetic material. Infecting a new bacterium can result in an exchange of bacterial DNA, this process is known as transduction and is the most common HGT mediated by viruses or viral particles.
Seeing a relative abundance as large as the one in the figures above lead us to think that is highly probable that HGT is happening in the cow's rumen. Whether ARGs are being transferred among bacteria via transduction or not is hard to tell but knowing that ARGs make up around 1% of the total genes found in the rumen it is logical to think that these genes might be introduced into new bacteria.

The second most abundant viruses found are the *Poxviridae*. This is a family belonging to the phylum *Nucleocytoviricota* formed by giant viruses (Adams et al., 2016). In this family, the genera *Parapoxvirus* and *Orthopoxvirus* can cause skin lesions in both humans and ruminants. Among *poxviridae* there are other viruses that need to be highlighted such as the Cowpox virus and the Bovine papular stomatitis virus which can infect via necrotic tissue or damaged skin and cause ulcerative lesions of the mucosae and skin (Scagliarini et al., 2016).
The third most abundant viruses in our samples are the *Mimiviridae*, a family that also belongs to the *Nucleocytoviricota* phylum (Adams et al., 2016). *Mimiviridae* are viruses associated to pneumonia in humans. These viruses can replicate in both human phagocytes (Raoult et al., 2007) and amoebas (Saadi et al., 2013). Being capable of infecting amoebae explains why we can find these viruses in the dairy cattle rumen.

16.3.5 Eukaryote

16.3.5.1 Phylum

Ciliates (*Ciliophora*) are protozoans characterised for the presence of cilia in their membranes. Cilia are organelles with sensorial or motor functions (Gao et al., 2016). This phylum often have symbiotic relationship with methanogenic archaea, meaning that a large RA of *Ciliophora* can be directly correlated with high methane emissions (Saborío-Montero et al., 2019). These eukaryotes are also involved in the biohydrogenation of fatty acids in the rumen (Francisco et al., 2019).

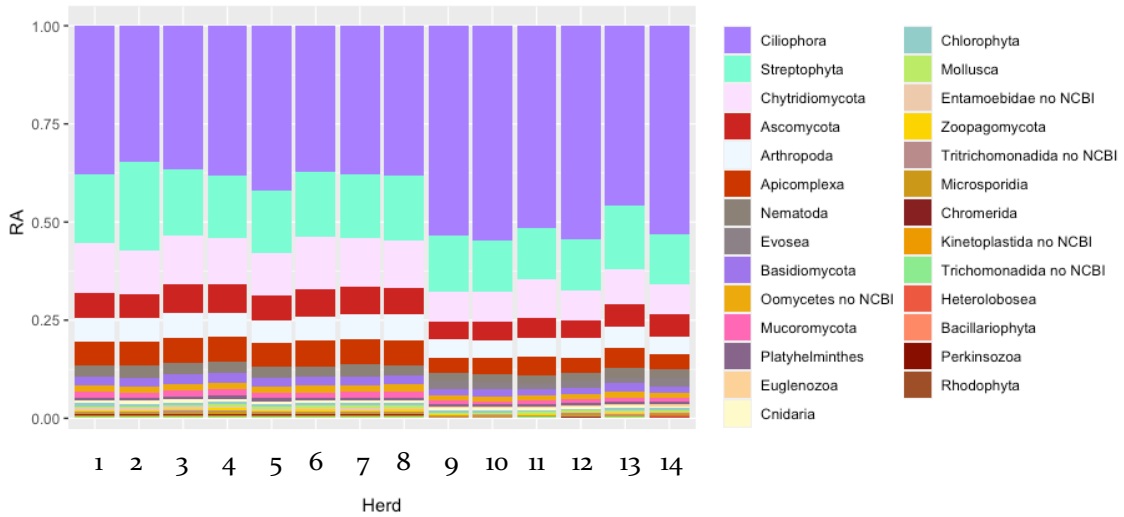

Figure 39 | **RA of the eukaryote phyla with a RA>0.05.**

Oligohymenophorea are a class of facultative parasites that often infect marine organisms (Lynn et al., 2000).

In the introduction we said that bacteria make up around the 90% of the rumen microbiota. In the superkingdom figure we have seen that in all the herds, the RA

was over 75%. We can say that our results fit with those described in the literature.

Figure 40 shows the genera of bacteria with a RA greater than 0.005%. We can see that *Prevotella*, *Butyrivibrio*, *Ruminococcus*, *Ruminococcacea* and *Lachnospiraceae* appear in the plot. All the genera which names start by Unc (unclassified) are those which genus cannot be assigned by NCBI. The name following "Unc", for example Unc Bacteroidetes is the highest taxonomical rank (phylum in this case) that can be assigned.

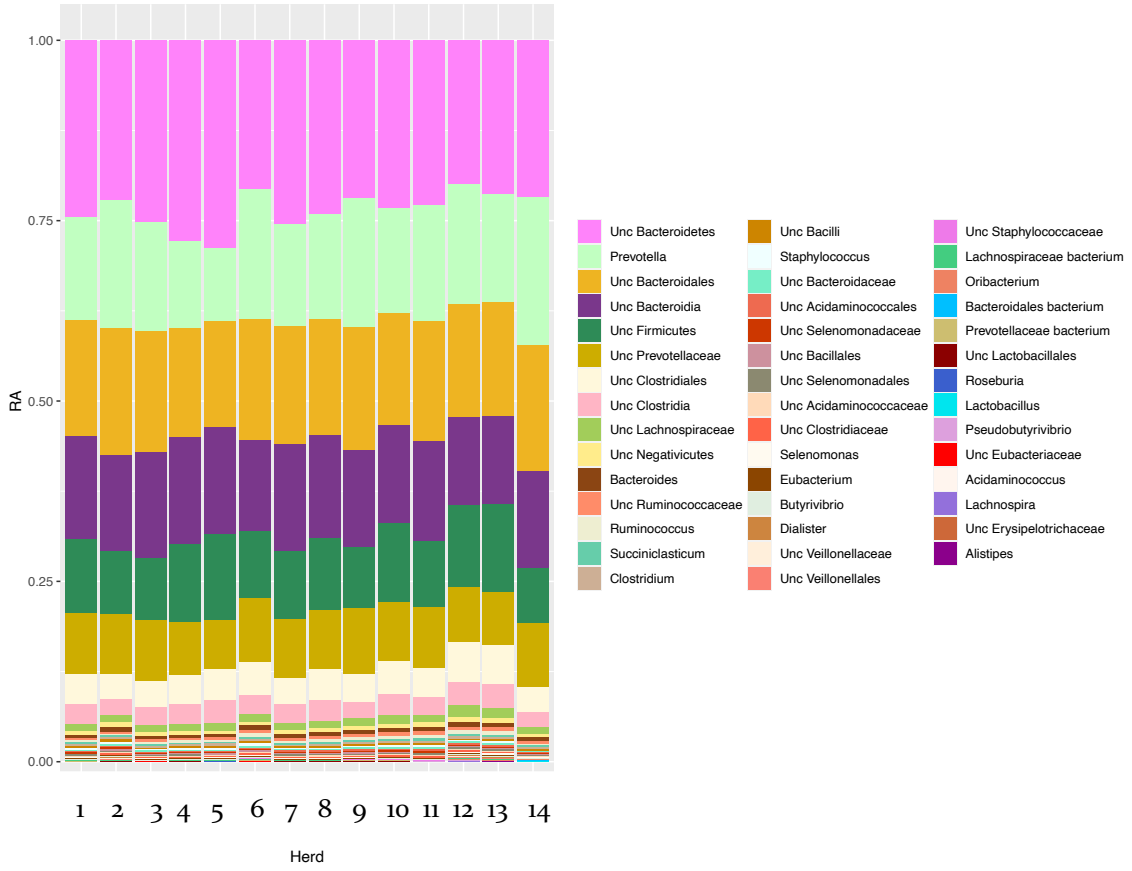

Figure 40 | **RA of bacteria genera.**

However, in the introduction we described that *Methanobrevibacter gottshalkii* and *Methanobrevibacter ruminantium* computed for 74% of all ruminal archaea (Henderson et al., 2015b; Weimer, 2015) but in our samples they are around the 10-15% of the ruminal archaea described. In figure 41 we see that most of the archaea found are methane related.

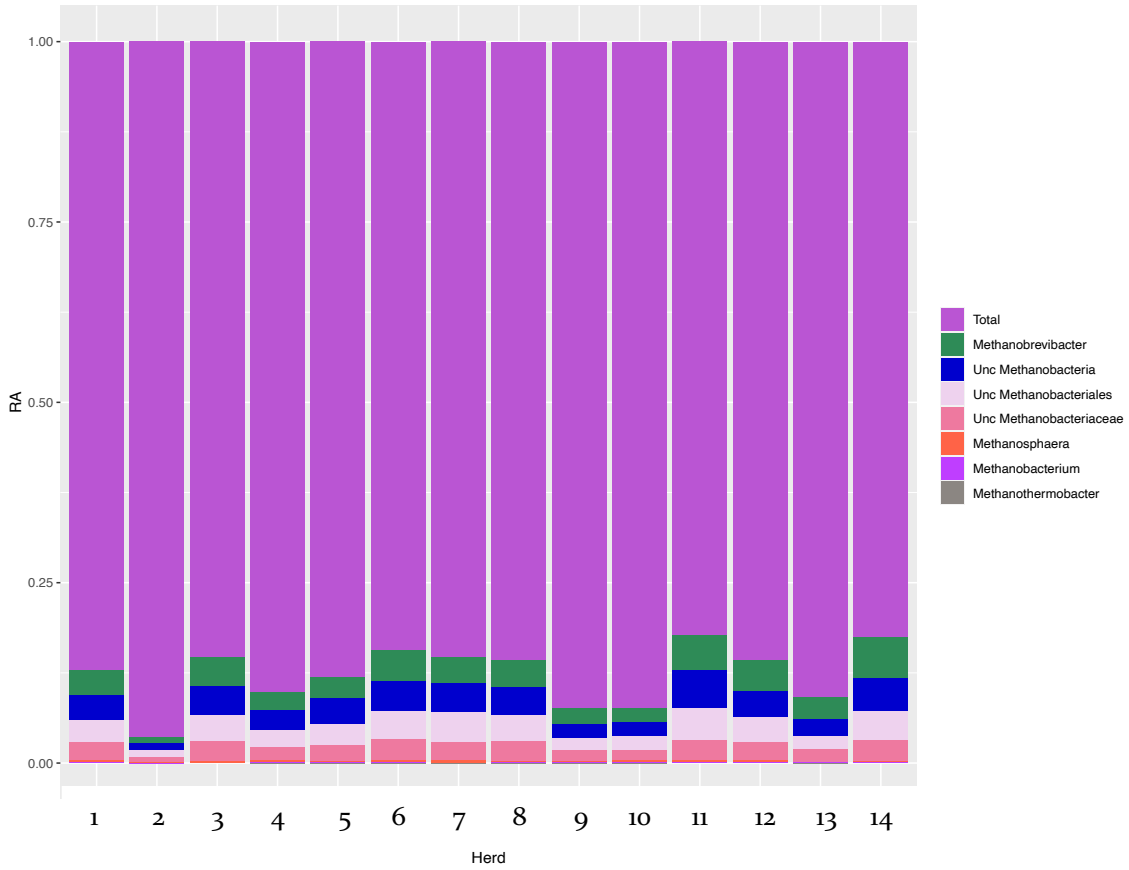

Figure 41 | **RA of the archaea species.** Archaea represented in purple are non-methane realated while the rest participate in the production of CH_4.

16.4 Calculating the relative abundance of antimicrobial resistance genes

Initially, the 20 most abundant genes of each herd were calculated and grouped. The list ended up having 25 genes which are the following: *tetQ, tetW, tetB(P), rpoB2, Staphylococcus mupB conferring resistance to mupirocin, Staphylococcus mupA conferring resistance to mupirocin, macB, cmeB, arlR, novA, lnuC, vatB, lmrD, bcrA, parY, ugd, optrA, oleC, efrA, efrB, msbA, tetA(58), TaeA, vmlR, Bifidobacterium adolescentis rpoB mutants conferring resistance to rifampicin*. Most of which had been described in the introduction based on the available literature.

Genes with a RA greater than 0.005% have also been studied, the 69 genes that meet this requirement are shown in table 3.

Gen	AMR Gene Family	DRUG Class	Resistance mechanism	Resistome (SPECIES)
arlR	major facilitator superfamily (MFS) antibiotic efflux pump	acridine dye, fluoroquinolone antibiotic	antibiotic efflux	Staphylococcus aureus
novA	ATP-binding cassette (ABC) antibiotic efflux pump	aminocoumarin antibiotic	antibiotic efflux	No prevalence data
Streptomyces rishiriensis parY mutant conferring resistance to aminocoumarin	aminocoumarin resistant parY	aminocoumarin antibiotic	antibiotic target alteration	No prevalence data
baeR	resistance-nodulation-cell division (RND) antibiotic efflux pump	aminocoumarin antibiotic, aminoglycoside antibiotic	antibiotic efflux	Escherichia coli, Klebsiella oxytoca, Salmonella enterica, Shigella dysenteriae, Shigella flexneri, Shigella sonnei
cmeB	resistance-nodulation-cell division (RND) antibiotic efflux pump	cephalosporin, macrolide antibiotic, fluoroquinolone antibiotic, fusidic acid	antibiotic efflux	Campylobacter jejuni
vanHO	glycopeptide resistance gene cluster, vanH	glycopeptide antibiotic	antibiotic target alteration	No prevalence data
vanRE	glycopeptide resistance gene cluster, vanR	glycopeptide antibiotic	antibiotic target alteration	No prevalence data
vanRF	glycopeptide resistance gene cluster, vanR	glycopeptide antibiotic	antibiotic target alteration	No prevalence data
vanRM	glycopeptide resistance gene cluster, vanR	glycopeptide antibiotic	antibiotic target alteration	No prevalence data
vanRO	glycopeptide resistance gene cluster, vanR	glycopeptide antibiotic	antibiotic target alteration	No prevalence data
vanRI	glycopeptide resistance gene cluster, vanR	glycopeptide antibiotic	antibiotic target alteration	No prevalence data
vanTG	glycopeptide resistance gene cluster, vanT	glycopeptide antibiotic	antibiotic target alteration	Clostridioides difficile, Enterococcus faecium, Streptococcus agalactiae, Streptococcus anginosus
vanXI	glycopeptide resistance gene cluster, vanX	glycopeptide antibiotic	antibiotic target alteration	No prevalence data
macB	ATP-binding cassette (ABC) antibiotic efflux pump	macrolide antibiotic	antibiotic efflux	Neisseria gonorrhoeae
oleC	ATP-binding cassette (ABC) antibiotic efflux pump	macrolide antibiotic	efflux pump complex or subunit conferring antibiotic resistance	No prevalence data
Bifidobacterium ileS conferring resistance to mupirocin	antibiotic resistant isoleucyl-tRNA synthetase (ileS)	mupirocin	antibiotic target alteration	No prevalence data
poxtA	ABC-F ATP-binding cassette ribosomal protection protein	oxazolidinone antibiotic, macrolide antibiotic, streptogramin antibiotic, tetracycline antibiotic, phenicol antibiotic, pleuromutilin antibiotic, lincosamide antibiotic	antibiotic target protection	Enterococcus faecalis, Enterococcus faecium, Staphylococcus aureus
evgS	resistance-nodulation-cell division (RND) antibiotic efflux pump, major facilitator superfamily (MFS) antibiotic efflux pump	penam, macrolide antibiotic, fluoroquinolone antibiotic, tetracycline antibiotic	antibiotic efflux	Escherichia coli, Shigella flexneri, Shigella sonnei
bcrA	ATP-binding cassette (ABC) antibiotic efflux pump	peptide antibiotic	antibiotic efflux	No prevalence data
bacA	undecaprenyl pyrophosphate related proteins	peptide antibiotic	antibiotic target alteration	Escherichia coli, Shigella dysenteriae, Shigella flexneri, Shigella sonnei
ugd	pmr phosphoethanolamine transferase	peptide antibiotic	antibiotic target alteration	Escherichia coli, Shigella sonnei
PmrF	pmr phosphoethanolamine transferase	peptide antibiotic	antibiotic target alteration	Escherichia coli, Shigella dysenteriae, Shigella flexneri, Shigella sonnei
lmrC	ABC-F ATP-binding cassette ribosomal protection protein	phenicol antibiotic, macrolide antibiotic, tetracycline antibiotic, streptogramin antibiotic, lincosamide antibiotic, pleuromutilin antibiotic, oxazolidinone antibiotic	antibiotic target protection	No prevalence data

tlrC	ABC-F ATP-binding cassette ribosomal protection protein	pleuromutilin antibiotic, tetracycline antibiotic, oxazolidinone antibiotic, streptogramin antibiotic, lincosamide antibiotic, phenicol antibiotic, macrolide antibiotic	antibiotic target protection	No prevalence data
efrB	ATP-binding cassette (ABC) antibiotic efflux pump	rifamycin antibiotic, macrolide antibiotic, fluoroquinolone antibiotic	antibiotic efflux	Enterococcus faecalis
vatB	streptogramin vat acetyltransferase	streptogramin antibiotic	antibiotic inactivation	Staphylococcus aureus, Staphylococcus epidermidis
vatE	streptogramin vat acetyltransferase	streptogramin antibiotic	antibiotic inactivation	*Enterococcus faecium*
vatH	streptogramin vat acetyltransferase	streptogramin antibiotic	antibiotic inactivation	No prevalence data
carA	ABC-F ATP-binding cassette ribosomal protection protein	streptogramin antibiotic, lincosamide antibiotic, tetracycline antibiotic, pleuromutilin antibiotic, oxazolidinone antibiotic, macrolide antibiotic, phenicol antibiotic	antibiotic target protection	No prevalence data
vmlR	ABC-F ATP-binding cassette ribosomal protection protein	streptogramin antibiotic, oxazolidinone antibiotic, macrolide antibiotic, pleuromutilin antibiotic, tetracycline antibiotic, lincosamide antibiotic, phenicol antibiotic	antibiotic target protection	No prevalence data
vgaE	ABC-F ATP-binding cassette ribosomal protection protein	streptogramin antibiotic, pleuromutilin antibiotic, macrolide antibiotic, oxazolidinone antibiotic, tetracycline antibiotic, phenicol antibiotic, lincosamide antibiotic	antibiotic target protection	Staphylococcus aureus
sul4	sulfonamide resistant sul	sulfonamide antibiotic	antibiotic target replacement	No prevalence data
tet(35)	ATP-binding cassette (ABC) antibiotic efflux pump	tetracycline antibiotic	antibiotic efflux	Vibrio parahaemolyticus
tet37	tetracycline inactivation enzyme	tetracycline antibiotic	antibiotic inactivation	No prevalence data
tet(44)	tetracycline-resistant ribosomal protection protein	tetracycline antibiotic	antibiotic target protection	Acinetobacter baumannii, Clostridioides difficile,Clostridium perfringens !LOWPREV
otr(A)	tetracycline-resistant ribosomal protection protein	tetracycline antibiotic	antibiotic target protection	No prevalence data
tetQ	tetracycline-resistant ribosomal protection protein	tetracycline antibiotic	antibiotic target protection	No prevalence data
tetT	tetracycline-resistant ribosomal protection protein	tetracycline antibiotic	antibiotic target protection	No prevalence data
tetW	tetracycline-resistant ribosomal protection protein	tetracycline antibiotic	antibiotic target protection	Clostridioides difficile, Enterococcus faecium, Klebsiella oxytoca
tetB P	tetracycline-resistant ribosomal protection protein	tetracycline antibiotic	antibiotic target protection	Clostridium perfringens, Clostridioides difficile. ! VERY LOW PREV
tet36	tetracycline-resistant ribosomal protection protein	tetracycline antibiotic	antibiotic target protection	No prevalence data
adeR	resistance-nodulation-cell division (RND) antibiotic efflux pump	tetracycline antibiotic, glycylcycline	antibiotic efflux	Acinetobacter baumannii
oqxB	resistance-nodulation-cell division (RND) antibiotic efflux pump	tetracycline antibiotic, glycylcycline, fluoroquinolone antibiotic, diaminopyrimidine antibiotic, nitrofuran antibiotic	antibiotic efflux	Citrobacter freundii, Enterobacter hormaechei, Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Salmonella enterica, Shigella flexneri, Shigella sonnei
smeR	resistance-nodulation-cell division (RND) antibiotic efflux pump	cephamycin, cephalosporin, aminoglycoside antibiotic, penam	antibiotic efflux	Stenotrophomonas maltophilia
adeJ	resistance-nodulation-cell division (RND) antibiotic efflux pump	diaminopyrimidine antibiotic, phenicol antibiotic, tetracycline antibiotic, rifamycin antibiotic, carbapenem, penem, fluoroquinolone antibiotic, macrolide antibiotic, cephalosporin, lincosamide antibiotic	antibiotic efflux	Acinetobacter baumannii
patA	ATP-binding cassette (ABC) antibiotic efflux pump	fluoroquinolone antibiotic	antibiotic efflux	Streptococcus pneumoniae
patB	ATP-binding cassette (ABC) antibiotic efflux pump	fluoroquinolone antibiotic	antibiotic efflux	Streptococcus pneumoniae
lmrD	ATP-binding cassette (ABC) antibiotic efflux pump	lincosamide antibiotic	antibiotic efflux	Listeria monocytogenes

lnuC	lincosamide nucleotidyltransferase (LNU)	lincosamide antibiotic	antibiotic inactivation	Campylobacter coli, Streptococcus agalactiae
LlmA 23S ribosomal RNA methyltransferase	Llm 23S ribosomal RNA methyltransferase	lincosamide antibiotic	antibiotic target alteration	No prevalence data
efrA	ATP-binding cassette (ABC) antibiotic efflux pump	macrolide antibiotic, fluoroquinolone antibiotic, rifamycin antibiotic	antibiotic efflux	Enterococcus faecalis
optrA	ABC-F ATP-binding cassette ribosomal protection protein	macrolide antibiotic, pleuromutilin antibiotic, phenicol antibiotic, oxazolidinone antibiotic, tetracycline antibiotic, streptogramin antibiotic, lincosamide antibiotic	antibiotic target protection	Enterococcus faecalis, Enterococcus faecium
Staphylococcus mupA conferring resistance to mupirocin	antibiotic resistant isoleucyl-tRNA synthetase (ileS)	mupirocin	antibiotic target alteration	Staphylococcus epidermidis
Staphylococcus mupB conferring resistance to mupirocin	antibiotic resistant isoleucyl-tRNA synthetase (ileS)	mupirocin	antibiotic target alteration	No prevalence data
msbA	ATP-binding cassette (ABC) antibiotic efflux pump	nitroimidazole antibiotic	antibiotic efflux	Escherichia coli, Klebsiella oxytoca, Salmonella enterica, Shigella dysenteriae, Shigella flexneri, Shigella sonnei
mtrA	resistance-nodulation-cell division (RND) antibiotic efflux pump	penam, macrolide antibiotic	antibiotic efflux	Mycobacterium tuberculosis
CRP	resistance-nodulation-cell division (RND) antibiotic efflux pump	penam, macrolide antibiotic, fluoroquinolone antibiotic	antibiotic efflux	Citrobacter amalonaticus, Enterobacter asburiae, Enterobacter kobei, Klebsiella pneumoniae, Proteus vulgaris, Yersinia pestis, Shigella sonnei
arnA	pmr phosphoethanolamine transferase	peptide antibiotic	antibiotic target alteration	Pseudomonas aeruginosa, Pseudomonas fluorescens
MexF	resistance-nodulation-cell division (RND) antibiotic efflux pump	phenicol antibiotic, diaminopyrimidine antibiotic, fluoroquinolone antibiotic	antibiotic efflux	Pseudomonas aeruginosa
srmB	ABC-F ATP-binding cassette ribosomal protection protein	phenicol antibiotic, oxazolidinone antibiotic, streptogramin antibiotic, pleuromutilin antibiotic, tetracycline antibiotic, macrolide antibiotic, lincosamide antibiotic	antibiotic target protection	No prevalence data
oleB	ABC-F ATP-binding cassette ribosomal protection protein	phenicol antibiotic, streptogramin antibiotic, lincosamide antibiotic, pleuromutilin antibiotic, macrolide antibiotic, oxazolidinone antibiotic, tetracycline antibiotic	antibiotic target protection	No prevalence data
TaeA	ATP-binding cassette (ABC) antibiotic efflux pump	pleuromutilin antibiotic	antibiotic efflux	No prevalence data
Bifidobacterium adolescentis rpoB mutants conferring resistance to rifampicin	rifamycin-resistant beta-subunit of RNA polymerase (rpoB)	rifamycin antibiotic	antibiotic target alteration, antibiotic target replacement	Streptococcus pneumoniae
rpoB2	rifamycin-resistant beta-subunit of RNA polymerase (rpoB)	rifamycin antibiotic	antibiotic target alteration, antibiotic target replacement	No prevalence data
tetA 46	ATP-binding cassette (ABC) antibiotic efflux pump	tetracycline antibiotic	antibiotic efflux	No prevalence data
tetB 46	ATP-binding cassette (ABC) antibiotic efflux pump	tetracycline antibiotic	antibiotic efflux	No prevalence data
tetA 60	ATP-binding cassette (ABC) antibiotic efflux pump	tetracycline antibiotic	antibiotic efflux	No prevalence data
tetB 60	ATP-binding cassette (ABC) antibiotic efflux pump	tetracycline antibiotic	antibiotic efflux	No prevalence data
tetA 58	major facilitator superfamily (MFS) antibiotic efflux pump	tetracycline antibiotic	antibiotic efflux	No prevalence data

Table 3 | **Genes detected in the ruminal microbiota with a prevalence higher than 0.005% with their gene family, class, resistance mechanism and most common bacteria that carry them.**

The gene found with the highest prevalence in all the herd (around 0.050%) was *macB,* a gene conferring resistance to macrolide antibiotics. Macrolides are commonly used in Europe to treat mastitis but have a very long-acting period, which only allows to a single administration, leading to low concentration of the active form, becoming a way to develop antimicrobial resistance (Pyörälä et al., 2014). In figure 42 we can observe the RA of the 25 genes more abundant by herd.

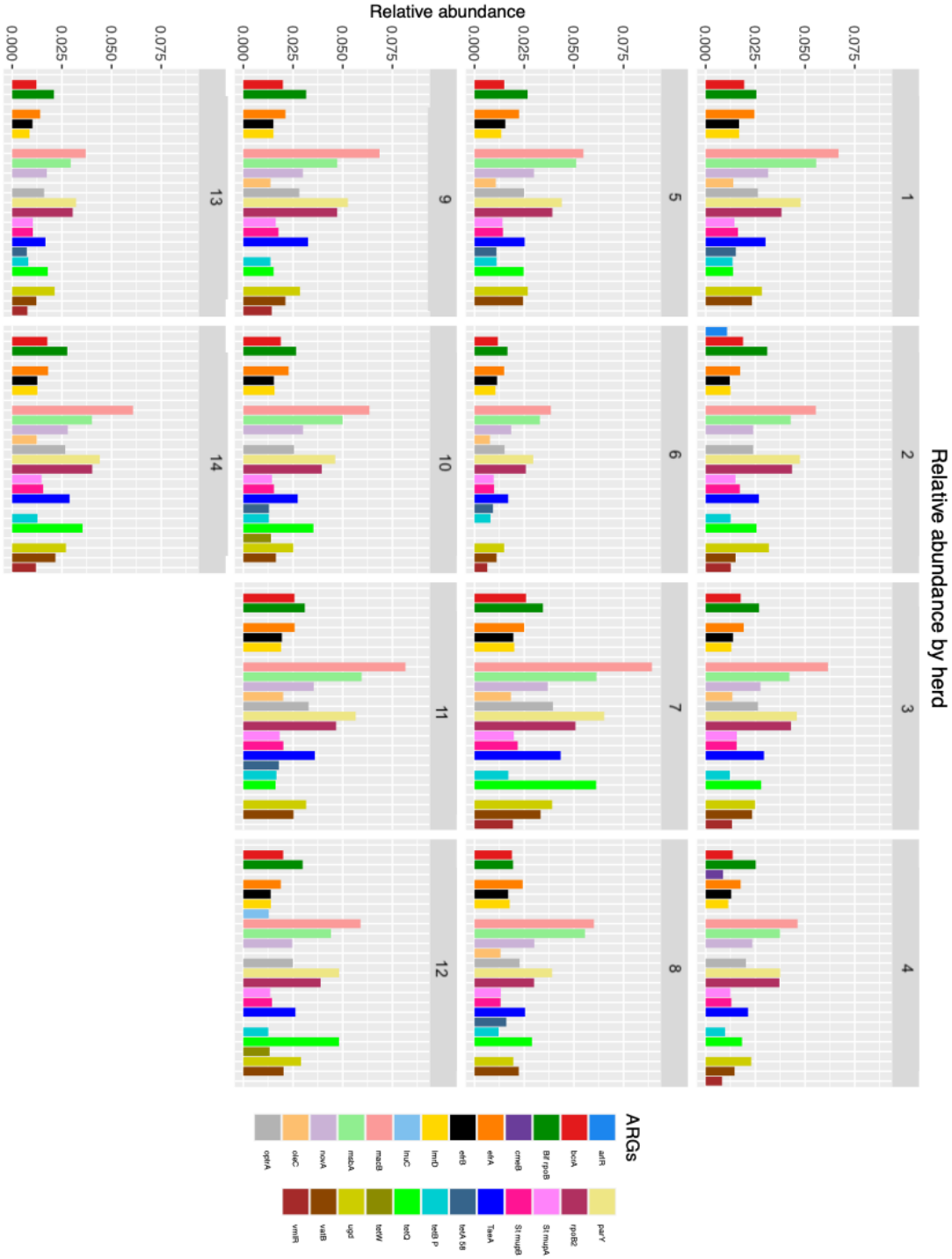

Figure 42 | **Relative Abundance (RA) of ARGs in each herd analysed.** Each box represents a different farm.

The high prevalence of tetracycline (TE) resistance genes is not surprising as it is one of the most common antibiotics used in veterinary medicine. It belongs to the group D, as it is a first-choice antibiotic. The main diseases in dairy cattle are mastitis and lameness, being the first-choice antibiotics those of the group of the beta-lactams, penicilins and cephalosporins.
The most abundant genes seem to be consistent both with the ones described in the literature and between the farms. In ovine rumen, the most prevalent genes found were *rpoC, gyrA/gyrB* and *tet37,* these genes confer resistance to rifamycin, aminocoumarin and tetracliclines, antibiotics used to fight mastitis. In our samples, the most prevalent genes *macB, masbA, pary, proB2, tetQ* and *TaeA* are also used to treat mastitis and lameness. Even though the genes are not the same, there is large similarity on the antibiotic group and the disease they treat. This is one of the expected results, as mentioned in the introduction, both mastitis and lameness are the two most common diseases in dairy cattle, so it was expected that resistance to these antibiotics were found.
As shown in the figure 42, the most prevalent genes were *macB*, *msbA*, *parY*, *rpoB2*, *tetQ* and *TaeA*. In table 4 we see that 4 of the 6 genes are related to the treatment of mastitis. The prevalence of msbA can be explained as nitroimidazoles were used as growth-promoters. Using antibiotics during more time than the desirable or when there is no infection leads to the appearance of resistances. Figure 43 shows the RA of these genes in each herd.

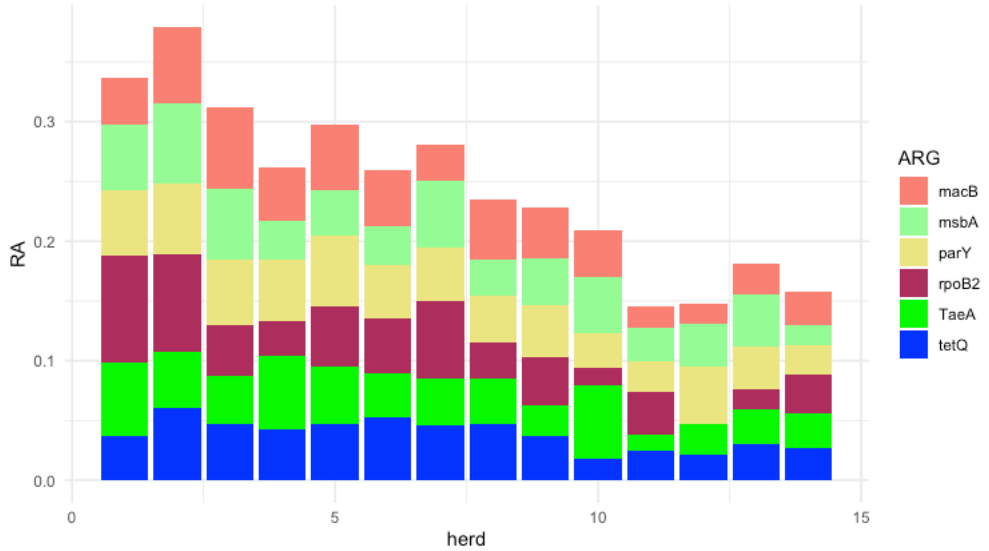

Figure 43 | **Relative Abundance (RA) of the 6 most prevalent ARGs by herd.**

Gene	Population RA	Antibiotic class	Mechanism	Disease
macB	0.06 ± 0.014	Macrolide	Efflux pump	Mastitis (Manual)
msbA	0.046 ± 0.01	Nitroimidazole	Efflux pump	Genital trichomoniasis in cattle. Bacterial and protozoa. Main growth-promoter. (Granja et al., 2013)
parY	0.045 ± 0.01	Aminocoumarin	Target alteration	Mastitis in dry dairy cattle (Amudson, 2005)
rpoB2	0.04 ± 0.007	Rifamycin	Target alteration and target protection	Mastitis (Redaelli et al., 1971)
tetQ	0.028 ± 0.013	Tetracycline	Target protection	Lameness and mastitis (Amudson, 2005)
TaeA	0.027 ± 0.007	Pleuromutilin	Efflux pump	Mycoplasma in swine (van Duijkeren et al., 2014)

Table 4 | **Six most abundant genes and their ATB information.**

The 69 most abundant ARGs of each farm were classified into 23 gene families. 23.99 % of them are related to "subunit of efflux pump conferring antibiotic resistance", 20.37% to "tetracycline resistant ribosomal protection protein", 10.92% to the vanR family, 5.21% to "ATP binding cassette ABC antibiotic efflux pump", 5.09% "rifamycin resistant beta subunit of RNA polymerase "rpoB" and to 4.93% "streptogramin vat acetyltransferase" amongst other as shown in the figure 44.

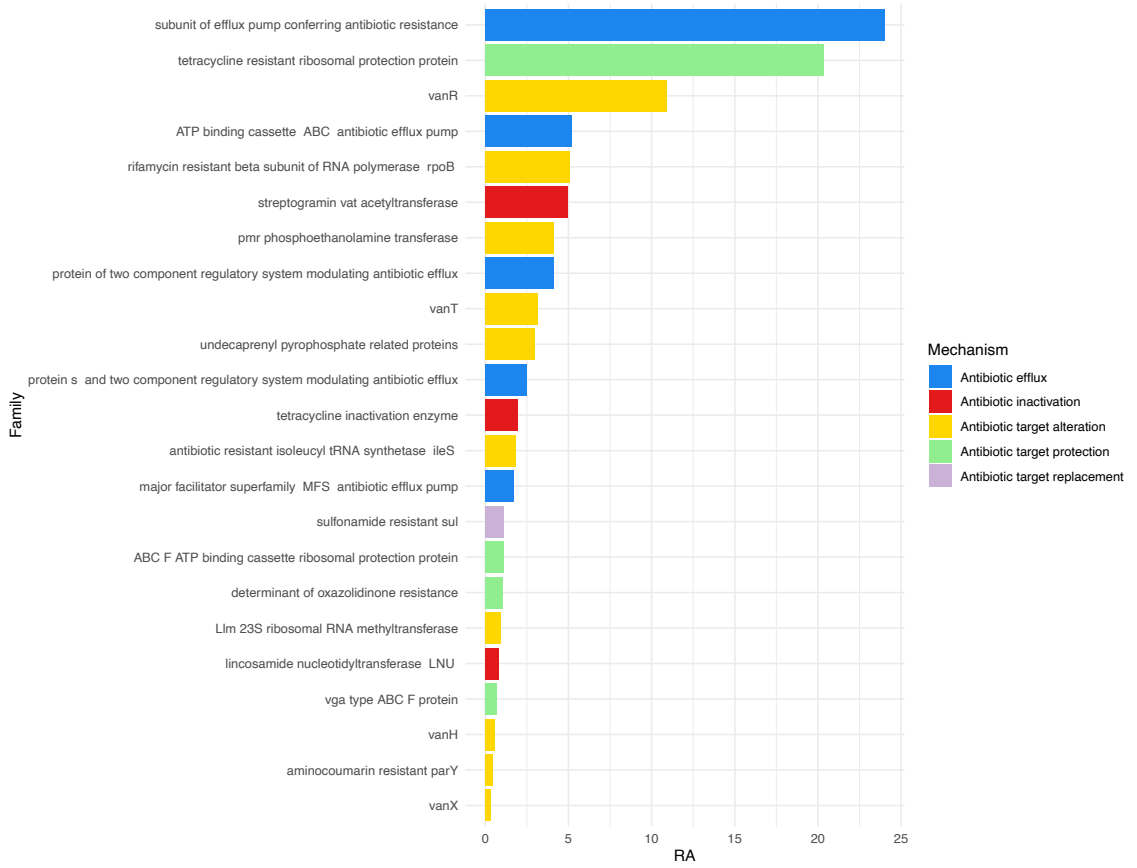

Figure 44 | **RA of the antimicrobial resistance families.** The colour represents the resistance mechanism associated to each family of ARM.

Around 25% of them belong to the efflux pump family, which means that the bacterium uses energy to eliminate the antibiotic. Processes and mechanisms mediated by ATP usually require of a selective pressure to be maintained, as evolution tends to remove processes that consume energy if they are not important to the survival of the microorganism.

Resistance to vancomycin is one of the most dangerous one, as this ATB is used as a last-line defence in life-threating infections mediated by Gram-positive bacteria. Vancomycin is used against methicillin-resistant Staphylococci and against Enterococci. If methicillin resistant bacteria gain resistance against vancomycin, there are very few alternatives to treat the infection. Staphylococci are already multi-resistance carries, being able to become a serious threat to human and animal health, especially in pets. This family of resistance can also be explained by the use of avoparcin, an analogous of vancomycin as a feed additive in livestock (Wijesekara et al., 2017).

Several AGRs detected are carried by pathogenic bacteria to humans and public health concern (*Clostridium difficile*, *Campylobacter jejuni, Salmonella Enterica, Neisseria gonorrhoeae*) and/or veterinary health (*Listeria monocytogenes, Staphylococcus aureus*), or by commensal and opportunistic bacteria as *E. coli, Enterococcus faecalis* and *Bacillus licheniformis* as well.

The distribution of ARGs between herds seems to be very similar and are compatible with the literature available. Antibiotic resistance genes *macB*, *msbA*

and *rpoB2* have the highest prevalence in all herds (*figure* 42). These genes are correlated with the resistance to macrolide, nitroimidazole and rifamycin antibiotics, respectively. ARGs related to tetracyclines were found in fourth position in the ranking.
We can fight antibiotic resistances using antibiotics with different action mechanisms knowing the most prevalent resistance families in each herd. If an antimicrobial target modification mechanism is very common in a farm, we can change its first election antibiotic to another one which cannot be affected by that mechanism before the bacteria become resistant to that antibiotic.

16.5 Category and family

It is desirable that most of the ARGs detected in our samples confer resistance to antibiotics associated to the Category C or D as these categories include first line treatments and ATB for which there are alternatives in human medicine. However, it does not seem to be the case, as a higher prevalence of category A and C was observed. It is worrying to see a RA that large of antibiotics of the category A as its use is strictly reserved to humans as they should not be used in food-producing animals and only in exceptional circumstances in companion animals.
Dairy calves do not drink their mother's milk in most cases, so the microbial vertical transfer only occurs during the delivery of the calf in the vaginal tract and in the early interaction with the environment.
It must be emphasised that cephalosporin (category B) are used to treat lameness and mastitis and tetracyclines (category D and larger RA of ARG) is not a first-choice treatment in dairy cattle. Carbapenems (category A) are not used in cattle as their use is restricted to human health, their presence could be explained by a transfer from the silage, from some additives directly provided in feed and soil microbiota.

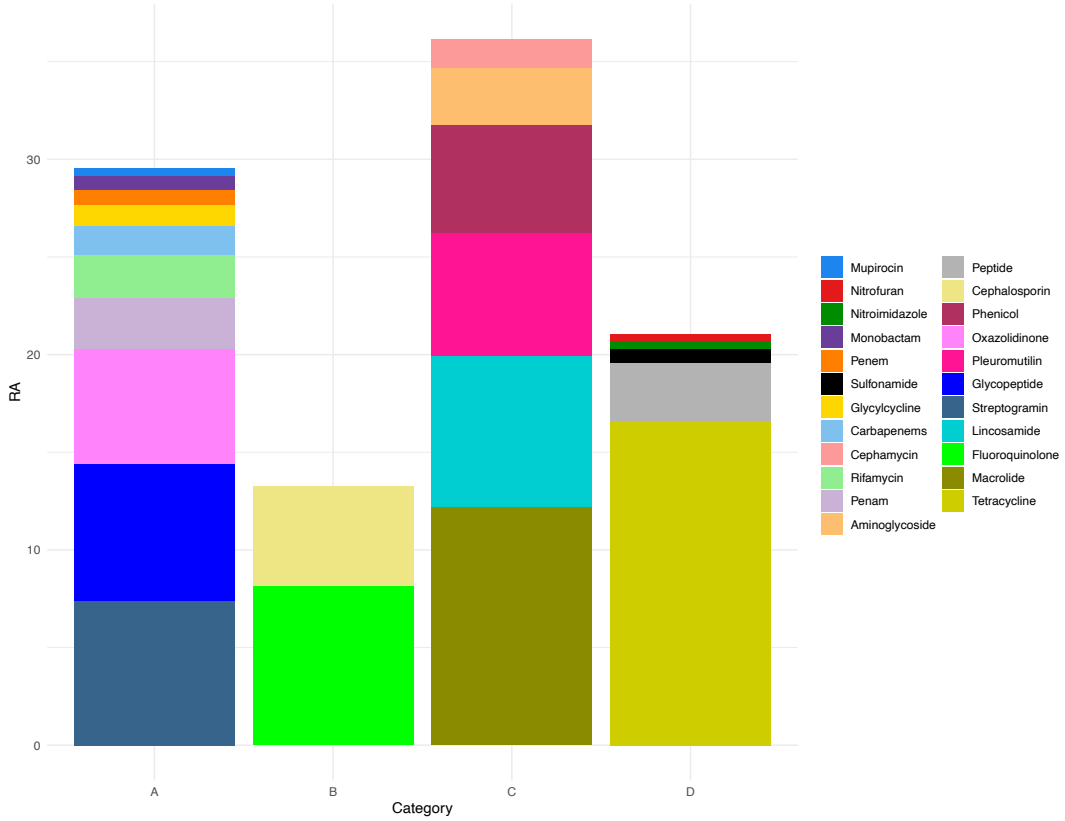

Figure 45 | **RA of the resistances to antibiotics.**

Antibiotics are rarely orally administered to dairy cattle. In most cases, they are administered by an intramammary or parenteral injection. A lot of antibiotics are extracted from bacteria that are usually part of the environment. The resistance can be obtained as a normal response of a bacterium to the toxin of another one and not only as a response to the use of the antibiotic. This could be the case of carbapenems, which are last-resort antibiotic used in human medicine but extracted from Enterococci and *Escherichia coli*.

The RA of category A and B antibiotic was similar (roughly 43%) to those in category C and D. This can be explained by the role of feeding and use of raw material as disseminators of antibiotic resistances. This could also be explained by the cross-resistances, a bacterium could firstly develop resistance to a category D antibiotic, but that same gene could confer resistance to antibiotics belonging to other categories because of chemical similarities of the molecule or by the mechanism of resistance. For instance, this is the case of the adeJ gene, which confers resistance to carbapenem, rifamycin, diaminopyrimidine, tetracycline, phenicol, penem, macrolide, lincosamide, cephalosporin and fluoroquinolone. This gene might have been gained originally as a defense mechanism against tetracycline antibiotics, commonly used in cattle which belongs to category D, and provides resistance to carbapenems, too.

These antibiotics are topically applied in hooves and intramammary-injected in the case of mastitis might get into the rumen by contaminated food or water or by licking the treated zone.

16.6 Bacteriophages correlation

The association between the abundance of ARGs and bacteriophages was studied, as the latter play a role in ARG transfer between bacteria. Figure 46 shows that the phenotypic correlation between the bacteriophages and the genes were positive and >0.40 with a p-value>0.05, supporting the hypothesis that bacteriophages are involved in the horizontal transmission of antimicrobial resistances.

It was also observed that most of the ARGs had large correlation between them, suggesting that there could be multi-resistant plasmid that usually carry those genes together. Legend of colours is the same as in the family of resistance figure. Most of these genes are involved in mechanisms of resistance of efflux pumps and target alteration.

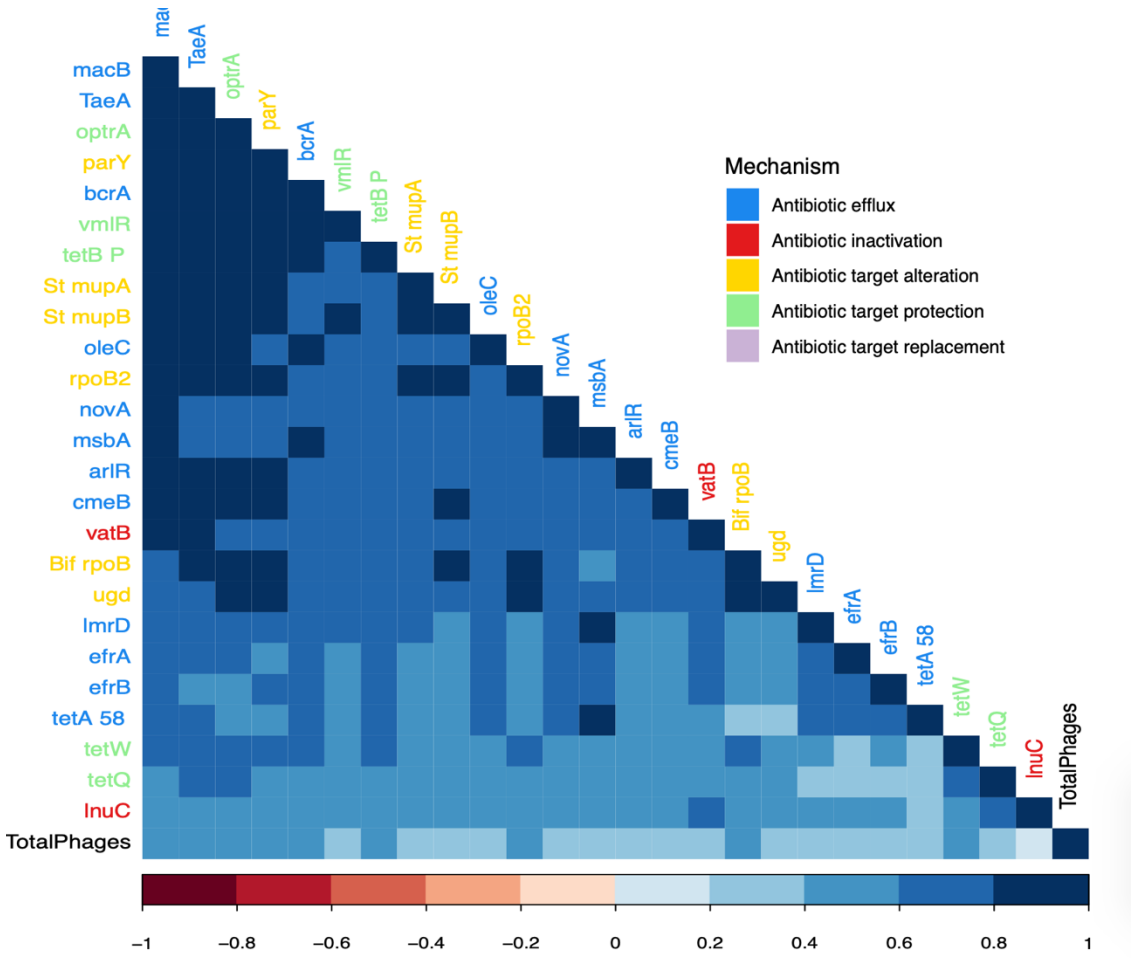

Figure 46 | **Correlation of ARGs with phages.** Colours in the names of the genes refer to the mechanism of resistance.

Among the 6 most prevalent genes, 4 of them were present in this cluster (*macB, parY, rpoB2 and TaeA*) which are related to mastitis treatment except *TaeA*. Although *msbA* is not present in this cluster, it was highly correlated with *macB*. It is possible, that these two most prevalent genes are also inherited or transmitted together. The resistance to nitroimidazoles provided by *msbA* was related to its use as a growth promoter, giving strength to the idea that these

genes were not inherited independently in bacteria, as the resistance to an antibiotic that is no longer administered is only conserved if it is present with another ARG in a plasmid, as evolution tends to remove anything that supposes an energetic cost if there is no a selective pressure. If these bacteriophages (mainly the caudovirales order) infect bacteria, it is likely that virus-mediated HGT occurs.

The high phenotypic correlation between both relative abundances we observed opens a new door to the use of antibiotic as phenotypic traits modulators, the use of these viruses as vectors to insert genes into the desired bacteria or using them as natural antibiotic, driving them into opportunistic or pathogenic bacteria to regulate the ruminal microbiota.

16.7 ARGs heritabilities

Heritabilities for the RA of the most prevalent ARGs were also estimated, resulting in a range from 0.10 to 0.49 with median of 0.18 and mean of 0.21 (*figure* 47).

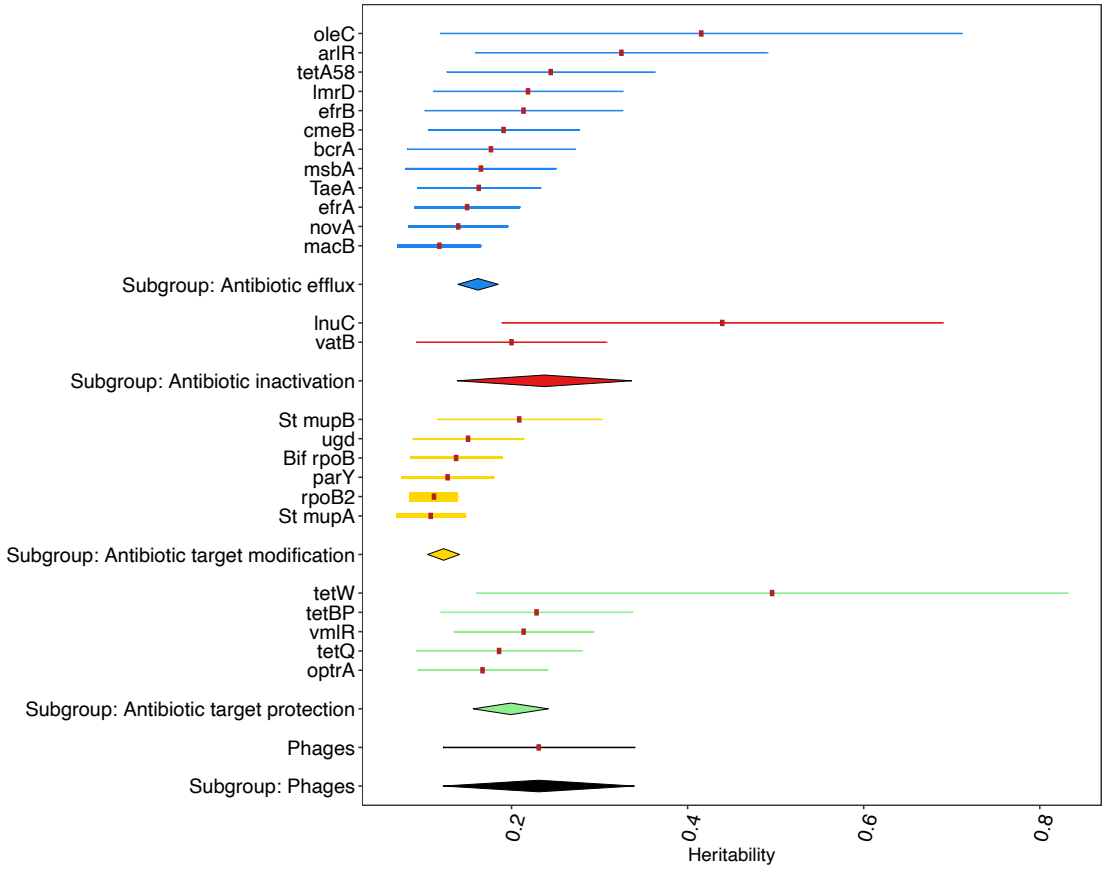

Figure 47 | **Heritability estimates for the relative abundance of the 25 most prevalent ARGs (by category) and for the RA of total phages.** The heritability values are represented by the red points and the HPD95 by the lines around them. The diamond represents the pooled result of that subgroup.

The ARGs with the lowest heritabilities were those that showed larger association with the abundance of bacteriophages. This could be because bacteriophages mediate the transfer of these genes among bacteria or because these genes are usually obtained by a multidrug resistance plasmid.
Large posterior standard deviation was observed for the heritability estimates in all ARGs, mainly caused by the small sample size in the study. A larger sample size would be necessary for more accurate estimates.
Relative abundance of bacteriophages had a heritability of 0.21, meaning that the RA of the bacteriophages partially depends on the genetic backgournd of the animals, and the presence of the ARGs could increase in each generation of cows favouring HGT to happen.

16.8 Correlations

Genetic correlation between the RA of ARGs and productive traits showed a wide range depending on the trait and the resistance gene (from -0.70 to 0.70). A negative genetic correlation (averaging -0.18) between ARGs and methane emissions was observed (*figure* 48). This makes sense as ARGs are usually carried in plasmids by bacteria, while the organisms responsible for the CH_4 emissions are archaea and protozoa. The more ARGs present in the rumen microbiota, the more bacteria displacing other microorganisms from their niches is expected. We speculate that the high correlation with methane emissions is because antibiotic resistances are mainly carried by bacteria in plasmids, these bacteria have an adaptative advantage over other microorganisms that inhabit the rumen such as fungi or protozoa, which are associated to larger CH_4 emissions as they provide substrate for methanogenic archaea.

Other productive traits like fat and protein yield did not show large correlation estimates (-0.05, 0.01 sd=0.004) with ARGs. Milk production appeared to be negatively correlated with most ARGs (averaging -0.22 with an standard deviation of 0.004). Fat yield and protein yield had null averaged correlation with ARGs. However, some ARGs showed correlations over 0.40 (absolute values) with these traits.

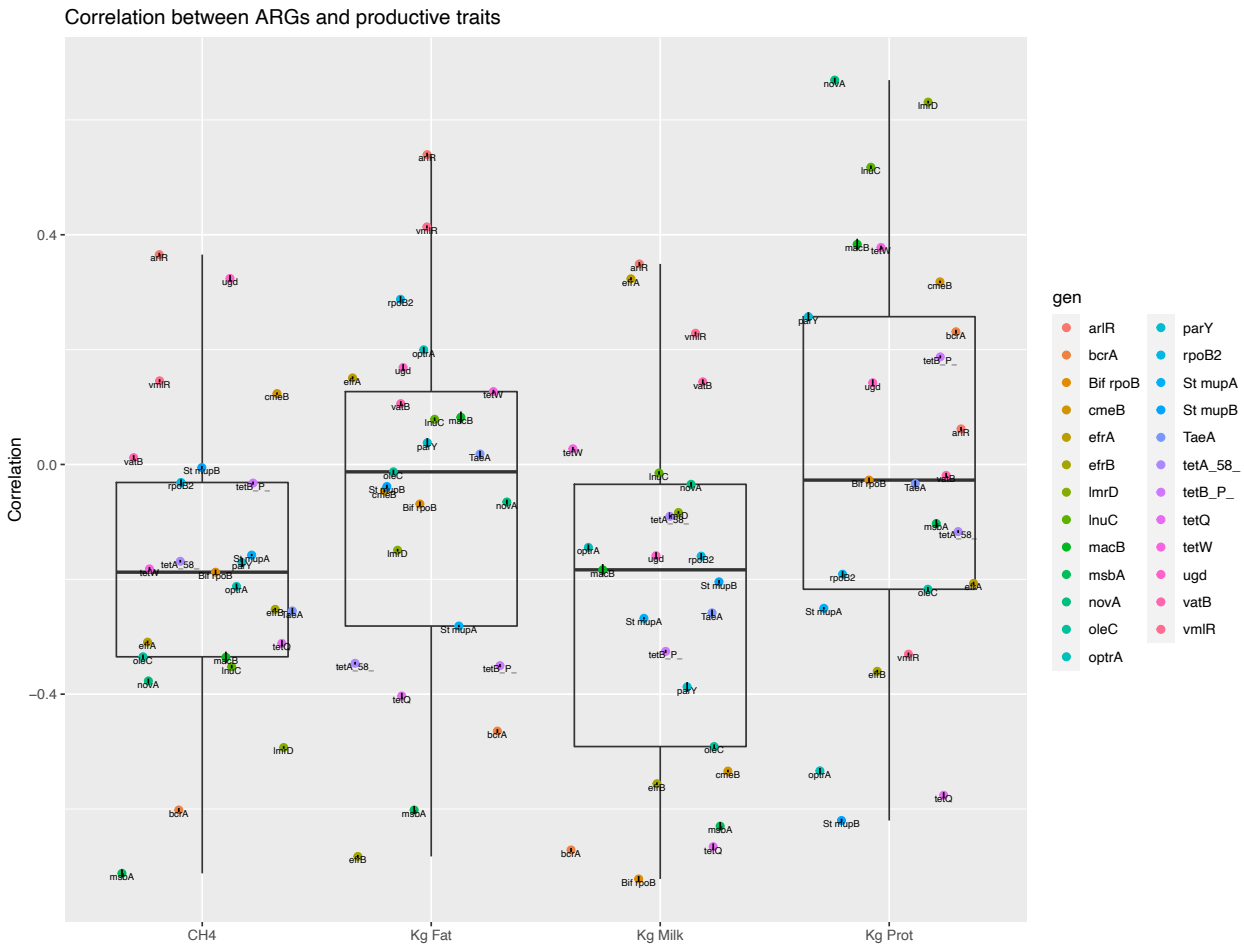

Figure 48 | **Correlation of ARGs with productive traits.** Each dot represents the correlation between a gene and a productive trait. The top of the box is the Q1 (25th percentile) and the bottom of the box corresponds to Q3 (75th percentile) while the line in the middle is the median. The end of the vertical lines is the maximum and the minimum values.

The gene *msbA*, related to the use of nitroimidazoles as growth promoter, was negatively correlated with all the traits. Even if this kind of practices were prohibited in Europe since 2006, we can still observe some effect of AMR resistances over the productive traits.

Tetracycline resistance genes showed negative correlation with the productive traits, especially with the kg of fat and milk produced. The larger the RA of these genes, the lesser milk production as the larger the RA the more mastitis-related treatments have been used on that animal. We observed that the interquartile range of both fat and protein yield were around 0, as well as their medians.

The real issue lies in the methane emissions and the milk yield, as these ARGs have a large effect on these traits. Resistant bacteria can displace other microorganisms from their niche and impact on the productive traits as we have observed. Reducing the amount of ARGs in the rumen could improve the productivity at the expenses of larger methane production. However, there were large variability on the genetic correlation between ARGs and these traits, and

further research needs to be done to differentiate between those having positive and negative correlations.

17 General discussion

The effects associated to the rising of antimicrobial resistant microorganisms are currently of main concern. Most of the zoonotic processes are caused by viruses that can affect both animals and humans, but antibiotics are frequently used to battle secondary infections. In the COVID-19 pandemic, the WHO has warned about how the misuse of antibiotics in COVID-19 mild cases could worsen the situation regarding AMR and to only use them when there are clear signs of bacterial infections (Bulletin of the World Health Organization | Enhanced Reader).

It needs to be taken into account that not every gene present in the rumen will be disseminated in the environment, but a larger RA of ARGs is expected to be present in the ruminal microbiota than in the gut or the faecal micriobiota. Also, that the ruminal ARGs have seldom be obtained by the selective pressure of antibiotics and may have been gained by HGT with environmental bacteria or by remainders found in food or surroundings. To get a deeper insight on how the resistances arrive into the rumen, it is necessary to have all information on the antibiotic usage in each farm with individualized records in different stages of the animal life.

This thesis shows that the role of the bacteriophages may be of interest as an indicator of ARG modulations, especially in early stages of the cow's development. The bacterial species that show favourable correlation with yield or sustainability traits could be selected by using bacteriophages that infect other bacteria that share the niche with these ones, leaving more space for them to grow, and thus improving the performance of the animal.

It is important to address these issues from One Health Initiative, and improve the communication and sharing of knowledge between animal health professionals, human health professionals and environmentalist. This work is focused on animal health, but under the scope of which genes are becoming a risk regarding human health. These resistances can jump to humans by indirect transmission from faeces used as manure for crops which could lead to the resistances passing to the plants and then to other animals or to us; or by direct transmissions for having contact with the animals or with some of their products. That is the reason why reducing or, at least controlling, the antimicrobial resistances that are appearing in livestock is also an improvement to human medicine.

18 Conclusion

1. Similar ARG were found in all farms in the study, as well as their RA, suggesting that ARGs are commonly present in dairy cattle.
2. The analysed ARGs have high phenotypic correlation between them, which could be a consequence of the presence of a multidrug resistant plasmid.
3. The most abundant ARGs were related to antibiotics used for mastitis and lameness. But ARG related to the past use of nitroimidazoles, formerly used as a growth promoter, was also found (e.g. msbA).
4. Among the genes with a prevalence greater than 0.005% analysed, 25% belong to the efflux pump family of resistance. Twenty percent of them were associated to resistance to tetracyclines, mediated by target protection and 15% to vancomycin resistance, an antibiotic belonging to category A and used as last-resort antibiotic in human medicine to treat intestine infections. The case of vancomycin needs to be carefully followed as can be a serious threat to human health.
5. There is a large relative abundance of category A antibiotics that have probably been obtained by cross-resistance. Carbapenems are for exclusive use in human medicine and its resistance can be explained by waste in silage, additives or contamination with soil microbiota. No antibiotic belonging to category A have been directly administered to these animals, as it is strongly prohibited.
6. Relative abundance of bacteriophages showed positive correlations with the ARG RA. This could be horizontal gene transfer of ARGs happening between ruminal bacteria. This hypothesis is supported by the large relative abundance of Caudoviriales, that make up almost the whole number of viruses found.
7. Knowing the antibiotic to which bacteria are most resistant to can help us to find alternatives before the risk increases. Personalised treatment for each farm could be implemented depending on the relative abundance of ARGs found in it.
8. The relative abundance of ARGs in the rumen microbiota showed heritability estimates ranging between 0.12 and 0.50. This suggests that the host genotype partially determines the abundance of those bacteria carrying ARGs.
9. The relative abundance of bacteriophages was also partially controlled by the host genotype, with heritability estimated at 0.21.
10. The relative abundance of ARGs showed negative genetic correlations with methane, suggesting that bacteria carrying ARGs can displace microorganisms associated with larger methane production.
11. The relative abundance of ARGs showed genetic correlations with production traits ranging from -0.40 to 0.40, and further research are needed to determine the role of ARGs and bacteriophages related to milk yield.

12. The genetic parameters estimated in this thesis showed some potential for selective breeding at modulating the presence of ARGs in the rumen microbiota.

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