YASMIN NEVES VIEIRA SABINO

EVIDENCE OF SELECTIVE PRESSURE AND HORIZONTAL GENE TRANSFER AMONG RUMINAL BACTERIA SUPPORT A HIGH PREVALENCE OF ANTIBIOTIC RESISTANCE IN THE RUMEN MICROBIOME

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Magister Scientiae*.

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Mateus Ferreira Santana

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(Orientador)

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BIOGRAPHY

YASMIN NEVES VIEIRA SABINO, filha de Jucilane Neves Vieira e Luís Antônio Sabino, nasceu em 22 de julho de 1993 e é natural de Senador Firmino - Minas Gerais.

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Ingressou no mestrado do Programa de Pós Graduação em Microbiologia Agrícola da Universidade Federal de Viçosa em fevereiro de 2017.

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ABBREVIATIONS LIST

AGPs - antibiotic growth promoters

AMR - antimicrobial resistance

ANVISA - Agência Nacional de Vigilância Sanitária

ARDB - Antibiotic Resistance Genes Database

ARGs - antibiotic resistant genes

ARGO - Antibiotic Resistance Genes Online

BEB- Bayes Empirical Bayes

BHI - Brain Heart Infusion

BV - Breakpoint value

CARD - Comprehensive Antibiotic Resistance Database

d_N/d_S - number of non-synonymous per synonymous substitutions

E-test - Epsilometer test

EU - European Union

EUCAST - European Committee on Antimicrobial Susceptibility Testing

FDA - Food and Drug Administration

GIT - gastrointestinal tract

HIV - Human Immunodeficiency Virus

ICE - integrative and conjugative element

iTOL – Interactive Tree of Live

JGI - Joint Genome Institute

KPC - *Klebsiella pneumoniae* resistant to carbapenemases

LRT - Likelihood Ratio Test Data

MDR - multidrug resistant

MGEs - Mobile genetic elements

MIC - minimal inhibitory concentration

MLS - macrolide, lincosamine and streptogramin

MRB - multidrug resistant bacteria

MRSA - methicillin-resistant *Staphylococcus aureus*

NCBI - National Center for Biotechnology Information

OD_{600nm} - optical density at 600nm

OiE - World Organization for Animal Health

PALM - Phylogenetic Analysis using Maximum Likelihood

RDP - Ribosomal Database Project website

RPKM - reads per kilobase in a gene per million mappable reads

SRA - Sequence Read Archive

US - United States

USA - United States of America

VRE - *Enterococcus* sp. vancomycin resistant

WGS – Whole genome sequencing

WHO - World Health Organization

ABSTRACT

SABINO, Yasmin Neves Vieira, M.Sc., Universidade Federal de Viçosa, February, 2019. **Evidence of selective pressure and horizontal gene transfer among ruminal bacteria support a high prevalence of antibiotic resistance in the rumen microbiome.** Adviser: Hilário Cuquetto Mantovani.

Infections caused by multidrug resistant (MDR) bacteria represent a therapeutic challenge both in clinical settings and in livestock production. Although antibiotics have been frequently used in cattle production, the distribution of antibiotic resistant genes (ARGs) in microorganisms that colonize the gastrointestinal tract (GIT) of ruminants is not well understood. In this study, we investigated the resistome of 435 genomes of ruminal bacteria and archaea and compared some of their phenotypes *in vitro* to their genotypes based on annotations of ARGs. We found that the number and classes of ARGs detected in ruminal bacteria genomes varied according to the computational tools used to search for antibiotic resistance. Genes encoding resistance to tetracycline were highly abundant/distributed in the genomes of ruminal bacteria and analysis of the d_N/d_S ratio indicated that the *tet*(W) gene in particular is under positive selective pressure. Our findings also revealed that the *tet*(W) gene is located in an novel integrative and conjugative element (ICE), suggesting a potential mechanism for horizontal transfer of tetracycline resistance in the rumen microbiota. Transcriptomic analyses showed that several ARGs are active in the rumen microbiome and were highly expressed in the GIT of humans. Some of the resistance phenotypes predicted *in silico* (31.5%) were also confirmed *in vitro*. Our data provide insight into the ARG profile of ruminal bacteria and reveal the potential role of mobile genetic elements in shaping the resistome of the rumen microbiome, with implications in human and animal health.

RESUMO

SABINO, Yasmin Neves Vieira, M.Sc., Universidade Federal de Viçosa, Fevereiro, 2019. **Evidence of selective pressure and horizontal gene transfer among ruminal bacteria support a high prevalence of antibiotic resistance in the rumen microbiome.** Orientador: Hilário Cuquetto Mantovani.

As infecções causadas por bactérias multirresistentes a antibióticos representam um desafio terapêutico tanto em ambientes clínicos como na produção pecuária. Apesar do uso frequente de antibióticos na produção de bovinos, a distribuição de genes de resistência a antibióticos em microrganismos que colonizam o trato gastrointestinal de ruminantes é pouco conhecida. Neste estudo, o resistoma de 435 genomas de bactérias e *archaea* ruminais foi investigado e alguns dos perfis de resistência identificados nesses genomas foram caracterizados *in vitro*. Os resultados mostraram que o número e as classes dos genes de resistência a antibióticos detectados nos genomas ruminais variam de acordo com as ferramentas computacionais utilizadas para a mineração dos genomas. Os genes que codificam resistência à tetraciclina foram abundantes nos genomas das bactérias do rúmen e a análise da relação d_N/d_S indicou que o gene *tet*(W), em particular, está sob pressão seletiva positiva. As análises também revelaram que o gene *tet*(W) está localizado em um novo elemento integrativo e conjugativo, sugerindo um mecanismo potencial para a transferência horizontal da resistência à tetraciclina na microbiota ruminal. Análises transcriptômicas mostraram que vários genes de resistência a antibióticos estão ativos no microbioma ruminal e também são expressos no trato gastrointestinal de humanos. Alguns dos fenótipos de resistência preditos *in silico* (31,5%) foram confirmados *in vitro*. Esse estudo expande os conhecimentos sobre o perfil de resistência a antibióticos em bactérias do rúmen e revela o papel potencial dos elementos genéticos móveis na modulação do resistoma do microbioma ruminal, com implicações para a saúde humana e animal.

GENERAL INTRODUCTION

Antibiotic resistance in microbial pathogens represents one of the greatest threats to human and animal health. The increasing number of resistant bacteria to the antimicrobial agents currently commercialized is frequently associated with the inadequate use and selective pressure imposed by these antimicrobials. Moreover, horizontal gene transfer represents one of the main factors contributing to the spread of antibiotic resistance genes (ARGs) in the environment. Therefore, there is an increasing concern about the lack of therapeutic alternatives to treat infections caused by multidrug resistant bacteria (MRB).

In this context, it is necessary to investigate the origin and reservoirs of the ARGs and evaluate how the resistant organisms and these ARGs are being transferred from one environment to another. One important target to study antimicrobial resistance is the animal gut microbiome, since the use of antibiotics for livestock production is intense. The rumen is the first compartment of the multicavitated stomach of ruminants (cattle, sheep, goats etc) and harbors a diverse anaerobic microbiota, composed by bacteria, protozoa, fungi, archaea and viruses. These microorganisms live in close physical contact, which facilitates the transfer of genetic material between different species. After weaning, the rumen can account for up to 80% of the digestive tract volume in cattle, and the flow of microbial biomass to distal parts of the gastrointestinal tract (GIT) results in the dissemination of these organisms (and their genes) in the environment through fecal excretion.

Antibiotic resistance in ruminal bacteria is not well documented and characterized, but some studies described this environment as a potential reservoir of ARGs. Antibiotics have been used by farmers at subtherapeutic doses as growth promoters in ruminants, and this practice may contribute to the selection and spread of antimicrobial resistance. It has been reported that the horizontal transfer of ARGs in conjugation assays increase when *Escherichia coli* (receptor strain) is mixed with a donor microbial community (e.g. sewage) before being inoculated into

medium containing antibiotic concentrations below the minimal inhibitory concentration (MIC). Based on the evidence that increased bacterial resistance was related to the use of antibiotics in livestock production, many countries in Europe and in other continents adopted rules to limit the use of antibiotics to compensate for poor sanitary conditions or to make animals grow faster, especially antimicrobials reserved for treatment of human infections.

Recently, hundreds of ruminal bacteria genomes were made available through the Hungate1000 Project (http://www.rmgnetwork.org/hungate1000.html), which allowed to hypothesize that genome mining could reveal the repertoire of antibiotic resistance genes in rumen microorganisms. Moreover, with the increasing concern about antibiotic resistance and the wealth of genomic data, many ARGs databases became available for *in silico* studies, making it possible to answer such questions. Therefore, this study aimed to: 1) identify and characterize the distribution of ARGs in genomes of ruminal microorganisms using different databases, 2) compare the results with the *in vitro* resistance phenotype of selected cultures, 3) evaluate the expression of these ARGs in metatranscriptome datasets of the rumen and the human gut and 4) study the genetic context, phylogenetic distribution and selective pressure of the detected ARGs. These data expands our knowledge about the importance of the rumen as a reservoir of antibiotic resistance genes and bring to light possible mechanisms mediating the transfer of ARGs from the rumen to the environment.

CHAPTER 1

BIBLIOGRAPHIC REVIEW

Microbial resistance to antibiotics

Antimicrobial resistance is a major economic and social problem that currently has raised the attention and concern of national and international governmental agencies, such as the Agência Nacional de Vigilância Sanitária (ANVISA), the World Health Organization (WHO) and the World Organization for Animal Health (OiE). In the United States (US) the infections caused by antibiotic-resistant bacteria account for $20 billion in excess healthcare costs, $35 billion in societal costs, and 8 million in additional hospital days[1]. Also, it has been estimated that approximately 50% of antibiotics used in clinical settings are prescribed unnecessarily to treat diseases that are not caused by bacteria, which represents an annual cost of US$1.1 billion[1,2]. The number of deaths associated with antibiotic resistance is increasing worldwide and is currently estimated in 700.000 cases per year[3].

The first report of antibiotic resistance dates back to 1940, when the first strain of penicillin-resistant *Staphylococcus aureus* appeared[4]. Since then, different microorganisms have been characterized as resistant to different antimicrobials (Figure 1). In some cases, the presence of resistant bacteria was detected before the antibiotic was introduced in the market[5], and it is noticeable that the selection of resistant bacteria often occurs faster than the development of antibiotics to fight them[6]. Currently, therapeutic alternatives to treat infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA), *Klebsiella pneumoniae* resistant to carbapenemases (KPC) and *Enterococcus* sp. vancomycin resistant (VRE)[7] are lacking.

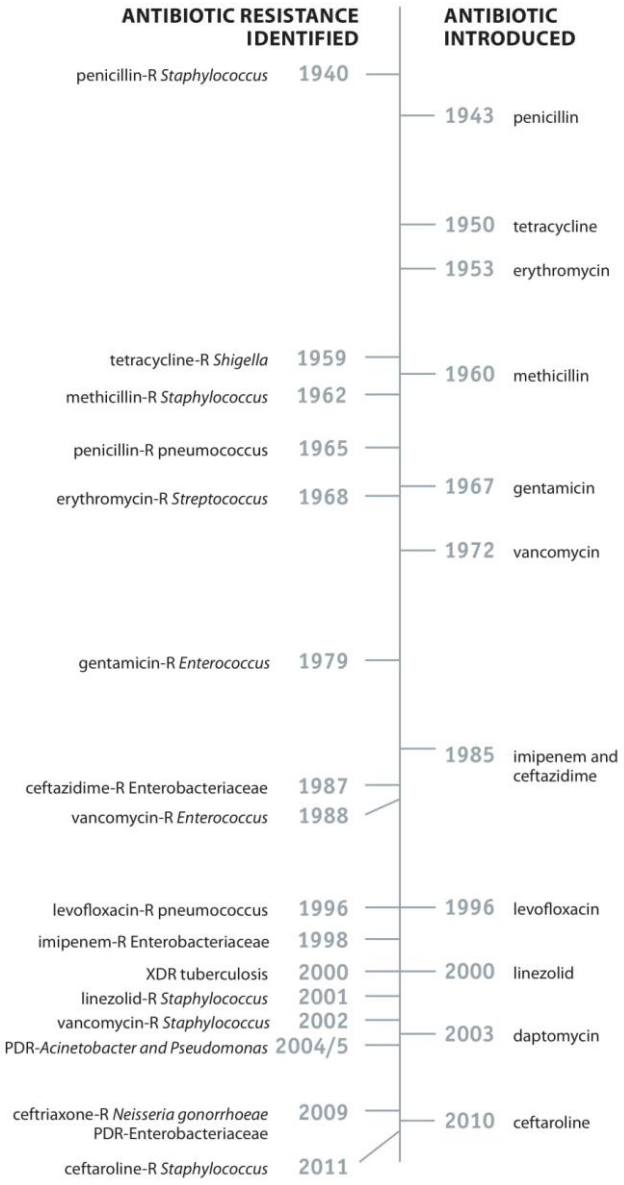

Figure 1. Schematic timeline showing the discovery/development of antibiotics and appearance of resistant strains[5].

The failure in therapeutic treatment of infections caused by multidrug resistant bacteria (MRB) is often caused by a diversity of resistance mechanisms. Resistant bacteria can cause enzymatic modifications or degrade the antibiotic, which reduces its activity or inactivates the molecule. Some strains produce compounds that protect the target site from the drug or express efflux pumps that reduce the intracellular concentration of the antibiotic. Additionally, resistant bacteria can modify the antibiotic target site or induce mechanisms that alter the

permeability of the cell membrane[6]. The resistance mechanisms can be intrinsic, when it is naturally and evolutionarily conserved, or acquired, as a result of mutations and events of horizontal gene transfer[8]. Mobile genetic elements (MGEs) such as insertion sequences, transposons, and gene cassettes/integrons are able to move within or between DNA molecules carrying ARGs in a cell. Other elements, such as plasmids and integrative conjugative elements (ICEs), can be transferred between cells[9], spreading the resistance in the bacterial population.

Insertion sequences and transposons are elements that can change their location in the genome due to the presence of the transposase gene, while integrons are genetic units that carry out site-specific recombination by rearranging ORFs located in gene cassettes[9]. Plasmids are circular mobile elements that replicate independently of the bacterial chromosome and can be transferred by conjugation to other cells. ICEs, also called conjugative transposons, are composed of genetic material that can be transferred vertically or horizontally, when in a host-integrated state or through excision and transfer to new recipients, respectively[9,10]. The transfer of antibiotic resistance genes (ARGs) by MGEs plays a major role in the dissemination of ARGs in microbial ecosystems[11]. Therefore, monitoring the distribution of ARGs in the GIT microbiota of livestock animals and humans could be useful to link antibiotic use in agriculture with resistance phenotypes among commensal bacteria and clinically relevant pathogens.

Use of antibiotics in agriculture

According to the Food and Drug Administration (FDA), several classes of antibiotics have been approved for use in food-producing animals, including compounds with clinical applications, such as aminoglycosides, cephalosporins, lincosamines, penicillins, macrolides, fluoroquinolones, sulfonamides and tetracyclines. Among them, tetracycline accounts for 70% of the total sales, followed by penicillins (10%), macrolides (7%), sulfonamides (4%), aminoglycosides (4%),

lincosamides (2%) and fluoroquinolones and cephalosporins (less than 1% each)[12]. Of these antimicrobials, 43% are sold for use in cattle, 37% intended for use in swine, 9% for turkeys, 6% for chickens and 4% in other species. Besides, 72% of the medically important antibiotics are administrated in the animal feed[13].

In 2014, the consumption of antimicrobials in the European Union (EU) by animals was more than two-fold greater than the consumption by humans (8,927 and 3,821 tonnes, respectively). Moreover, the population-weighted mean consumption of tetracyclines was 3.6 mg per kg of estimated biomass in humans and 50 mg per kg in food-producing animals. In addition, the use of antibiotics for livestock production appears to be increasing outside the USA and the UE, especially in countries such as China, India and Brazil[14]. Estimates suggest that antimicrobial use for production of swine, poultry, and cattle in Brazil, as well as in Russia, India, China, and South Africa, will double by 2030, compared to 2010[15].

Therefore, based on the potential selection of resistant bacteria associated with the use of antimicrobials in the livestock, several European countries have banned the use of antibiotics for growth promotion. Sweden pioneered the efforts to contain antimicrobial resistance in food-producing animals. They adopted strategies including regulated sales of antibiotics and enacted a ban on farm use of antibiotic growth promoters (AGPs). Other countries of the EU followed this initiative and also started to ban the use of AGPs in food animal production. Between 1972 and 1974, European prohibited the use of tetracycline, penicillin and streptomycin as growth promoters. In 1997, the EU banned avoparcin for all uses in agriculture. In 1999, EU officials discontinued the use of AGPs belonging to drug classes that were also used in human medicine, such as tylosin, spiramycin, virginiamycin and bacitracin. By 2006, the EU had banned all AGPs[16] from livestock production systems.

Currently, AGPs are still being extensively used in countries that are major meat producers (poultry, pig and cattle), such as China, Russia, India, Argentina, Indonesia, Philippines, South Africa and Brazil[14]. Although Brazil still allows the use of AGPs for livestock production,

including tilosin, virginiamycin and bacitracin, some antimicrobials such as tetracyclines, penicillins, chloramphenicol and systemic sulphonamides have been banned as growth promoters[17]. Due to the evidence and consistent reduction in the probability of detecting bacteria that are susceptible to antibiotics as the amount of antimicrobials consumed increase[18], alternatives to the antibiotics use as growth promoters has emerged. This includes greater investment in animal vaccination and the use of probiotic organisms[19], aiming to decrease the selective pressure on resistant bacteria and thus, reduce the risks of infections caused by MDR organisms in both animals and humans.

Risks to human health inherent of the use of antibiotics in animals

The resistance to antibiotics in humans could be inter-linked with the presence of resistance genes in other organisms, such as farm animals[20]. Analysis of whole genome sequencing (WGS) data suggests, for example, that the MRSA lineage CC97 has entered in the human population from a livestock source[21]. Additionally, analysis of concatenated multi locus sequence data showed that MRSA lineage ST5 disseminated globally in poultry after a cross-species transmission from humans[22]. Another report demonstrated resistance to carbapenems in bovine enterobacteria, despite the fact that this class of antibiotic is not allowed for use in farm animals. The authors suggested that the resistance was acquired from another source, probably humans, but a direct cause-effect relationship was not evident from their study[23]. Moreover, it was reported a decrease in the incidence of vancomycin-resistant *Enterococcus* in bacteria isolated from poultry meat and faecal samples from humans following the discontinuation of avoparcin (vancomycin-like molecular structure) use in animals[24]. These data provide insights of the correlation between bacterial resistance in humans and animals, demonstrating that the use of antibiotics in production animals may influence the pattern of resistance observed in humans.

Some studies also report that antibiotic resistance can be transferred from animals to humans due to the ingestion of foods, contaminated water, or from the direct contact between farmers and rural workers with animals or their feces[25,26]. A previous study correlated the spread of livestock-associated methicillin-resistant *S. aureus* (LA-MRSA CC398) to an increased number of human infections and attributed this to the animal movements in the field[27]. This movement could have a critical role in the dissemination of strains, although the possibility of transference by other routes, such as contaminated fomites, cannot be discarded. Moreover, MGEs propitiate the transference of ARGs between bacteria from animals and humans. For example, a class I integron carrying ARGs was identified in *E.coli* and *Salmonella* isolated from humans, with an identical gene cassette found in diverse animal bacterial species, including *E. coli* from food-producing animals[28]. Besides, the detection, for the first time, of a colistin resistance gene (*mcr*-1) in a plasmid of *E. coli* isolated from pigs and human isolates provide evidence for the spread of *mcr*-1 from animals to humans[29].

The FDA has proposed several strategies to prevent the selection of antibiotic resistant bacteria in agricultural practices and to avoid the possible transfer of resistance genes from farms to humans. These recommendations include limiting the use of medically important antibiotics in livestock only to strictly necessary cases and under the supervision of a veterinarian. Thus, with the judicious use of clinical relevant antibiotics in livestock, resistance selection pressure should decrease and the effectiveness of these drugs for use in clinical practice may increase[13].

Some alternatives to overlap the low efficacy of the available antibiotics in front of multi-resistant bacteria have been proposed, such as the design of antimicrobial peptides with broader spectrum of activity[30], the investment in phage therapy[31], the utilization of drug combination therapy[32], the use of nanoparticles to control the release of antimicrobials *in vivo*[33] and the modification of conventional antibiotics[34]. Other suitable options to overcome the antibiotic resistance threat are the discovery of

novel targets in the bacterial cell using genomic approaches and the design of molecules that affect the mechanism of infection, including drugs that block attachment of bacteria to biotic and abiotic surfaces[35]. To better evaluate the possible targets of new drugs as well as to develop strategies to avoid the dissemination of AMR, it is important to investigate the genetic determinants causing AMR and the potential sources of resistance.

Antibiotic resistance in ruminal bacteria genomes/cultures

Some studies have investigated the occurrence of antibiotic resistance in the ruminal ecosystem[36-38]. Among the impact caused by the use of antibiotics in livestock, it was demonstrated that these drugs can modify the host microbiota, affect immune responses and alter the function of the gastrointestinal tract[39,40]. In the rumen environment, microorganisms are essential to digest complex polysaccharides and to convert substrates into volatile fatty acids that are absorbed through the rumen wall. A previous study demonstrated that antibiotics at therapeutic and sub-therapeutic concentrations alter the prevalence of specific bacteria in the rumen of cattle[37]. Furthermore, the rumen was recently described as a reservoir of antibiotic resistance in ovine and cattle, with potential implications to human and animal health[36,38]. The rumen shows high population density of microorganisms and high genetic diversity of microbial species, in addition to feed particles that provide surface area for direct physical contact between different species of microorganisms, facilitating events of horizontal gene transfer[41].

The study of antibiotic resistance in ruminal bacteria was facilitated with the launch of the Hungate1000 Project and the availability of online databases containing antibiotic resistance data. This project contains metadata of 501 microbial genomes, distributed in 9 phyla, obtained from 15 livestock sources from 21 countries[42]. This resource represents a goldmine of sequencing data for screening studies based on computational analysis. With the increasing number of genomes and metagenomes available in online databases and the growing urgency for

studies regarding the origins of antibiotic resistance, different tools have been implemented to identify antibiotic resistance genes in genomic sequences.

These resources include the Antibiotic Resistance Genes Online (ARGO), a beta-lactam and vancomycin database[43]; MvirDB, related to microbial protein toxins, virulence factors and antibiotic resistance genes[44]; Antibiotic Resistance Genes Database (ARDB), which unifies most of the publicly available antibiotic resistance genes[45]; Resfinder, the most current platform available, which identifies acquired resistance genes[46]; Comprehensive Antibiotic Resistance Database (CARD), that feature a unique organization of orthologous terms to antibiotic resistance[47,48]; ARG-ANNOT, developed to detect existing genes and, supposedly, new antibiotic resistance genes in bacterial genomes[49]; and Resfams, which differs from most search methodologies that uses Blast to identify resistance genes, relying on Markov chains to identify protein profiles associated with antibiotic resistance[50].

In addition to characterizing the reservoirs of antibiotic resistance genes, it is necessary to evaluate if they are being expressed *in vivo* and to define which conditions affect this expression. Previously, it has been demonstrated that the diversity and abundance of antibiotic resistance genes in beef cattle rumen varied according to the diet provided to the animal, being higher in concentrated-fed animals when compared to the forage-fed animals[36]. However, the impact of antibiotic use in ruminants in the acquisition and expression of ARGs is not well document. Therefore, it is necessary to characterize the abundance of ARGs in the rumen microbiome and to investigate potential mechanisms that could mediate the transfer of ARGs between commensal and pathogenic organisms.

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CHAPTER 2[1]

Title: Selection pressure and mobile genetic elements shape the antibiotic resistome in the rumen microbiome

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Abstract

Infections caused by multidrug resistant bacteria represent a therapeutic challenge both in clinical settings and in livestock production, but their prevalence in the gastrointestinal tract (GIT) of ruminants is not well understood. We investigated the resistome of 435 ruminal microbial genomes *in silico* and confirmed representative phenotypes *in vitro*. We found a high abundance of genes encoding tetracycline resistance and evidence that the *tet*(W) gene is under positive selective pressure. Our findings revealed that in several ruminal microbial genomes *tet*(W) is located in a novel integrative and conjugative element (ICE). Analyses of rumen and human faecal metatranscriptomes showed that antibiotic resistant genes (ARGs) are active in the GIT of animals and humans. Our data provide insight into the ARGs profile of ruminal bacteria and reveal the potential role of mobile genetic elements in shaping the resistome of the rumen microbiome, with implications in human and animal health.

Keywords: rumen microbiota, resistome, ARG, tetracycline resistance, *tet*(W), ICE

Introduction

Without immediate action to tackle the current and escalating threat of antimicrobial resistance (AMR), it is estimated that resistant infections may kill one person every three seconds by the year 2050, raising the death toll worldwide to 10 million annually[1]. *Staphylococcus aureus* and *Enterococcus* sp., together with members of the family *Enterobacteriaceae* (especially *Klebsiella pneumoniae*), *Pseudomonas aeruginosa* and *Acinetobacter* sp., are considered the greatest microbial threat to human health in clinical settings[2,3]. Each year, strains of methicillin-resistant *S. aureus* (MRSA) kill more Americans than Human Immunodeficiency Virus (HIV), Parkinson, emphysema and homicides combined[4]. This antibiotic resistance crisis can be linked to several issues, including the overuse of these compounds for medical and agricultural purposes, inadequate prescriptions and limited discovery/development of novel and effective antibiotics[5].

The widespread use of antibiotics in the agricultural sector plays an underrepresented role in the AMR context. Large amounts of antimicrobials are frequently used in livestock to prevent diseases and promote animal growth[6]. Approximately 15 million kilograms of antibiotics are sold annually in the United States of America (USA) for use in food-producing animals and about 60% of these compounds also have therapeutic applications in humans[7]. Previous studies indicate the co-occurrence of antibiotic resistant bacteria in humans and food animals[8,9], and a positive correlation was reported between the use of tetracycline in livestock and the increase of tetracycline resistance in *Salmonella* spp., *S. enterica* Typhimurium and *Clostridium jejuni* isolated from humans[10]. Genes encoding resistance to colistin, an important last-resort antibiotic used for the treatment of multidrug resistant (MDR) Gram-negative bacteria in clinical settings, have been identified in bacteria isolated from humans and animals in some countries where it has been used as a feed additive[11]. MDR bacteria have also been isolated from ruminants, which represent a potential reservoir of antibiotic resistance genes (ARGs)[12,13].

The rumen ecosystem is colonized by complex and genetically diverse microbiota that live in close physical contact, which could facilitate the exchange of genetic material between members of the microbiota and organisms that transit in the ruminant gastrointestinal tract (GIT)[14,15]. In addition, feeding antimicrobials through ruminant diets can impose a selective pressure on resistant organisms, potentially modifying the autochthonous ruminal microbiota[16]. These findings corroborate with recent analyses of the ovine rumen resistome, which identified resistances to 30 known antibiotics, including high abundance of genes for daptomycin and colistin resistance, two clinically relevant antibiotics[13].

Ruminants represent a major source of animal protein for human consumption worldwide (through milk and meat production) and are raised in close proximity with humans. Nonetheless, the occurrence and distribution of ARGs among ruminal bacteria is not well documented and the potential mobility of the ARGs within the ruminal microbiota has not been investigated. Hundreds of reference genomes of cultured ruminal bacteria and archaea have recently been made available through the Hungate Project (http://www.hungate1000.org.nz/), representing approximately 75% of the genus-level bacterial and archaeal taxa present in the rumen[17]. We therefore hypothesized that genome mining of the Hungate genomic resources could reveal the distribution and genetic context of ARGs within major ruminal species represented in the core rumen microbiome.

As such we analyzed 435 genomes of ruminal bacteria and archaea to search for ARGs using the ResFinder[18], ARG-ANNOT[19] and Resfams databases[20]. Identified ARGs were also evaluated for selective pressure, genetic context as well as their expression in different metatranscriptome datasets. The resistant phenotype was confirmed for some cultured representative of ruminal bacteria from the sequenced Hungate1000 collection using *in vitro* testing. Our findings shed light on the distribution and mechanisms of antibiotic resistance among rumen microbes, thereby improving our understanding about the risks associated with the unnecessary use of medically important antibiotics in food-producing animals.

Results

Detection of ARGs in ruminal microbial genomes using computational tools

Analysis of ARGs in rumen microbial genomes using ResFinder, Resfams and ARG-ANNOT showed differences in the number and classes of ARGs that could be detected by each computational tool. ResFinder identified a total of 141 acquired ARGs distributed across 11 antibiotic classes, ARG-ANNOT detected 754 genes across 10 antibiotic classes and Resfams predicted 1019 sequences related to the resistance of 8 classes of antibiotics. The majority of the genes detected by the three databases (726 genes) were related with resistance to beta-lactams, followed by glycopeptides (510), tetracycline (307) and aminoglycosides (193). Resfams detected 81.3% and 80.8% of the aminoglycosides and glycopeptides resistance genes, respectively, while ARG-ANNOT identified the majority (61.4%) of the beta-lactam resistance genes. Fluoroquinolone, fosfomycin, nitroimidazole and trimethoprim were the classes of antibiotics with the lowest number of ARGs identified using ResFinder, Resfams and ARG-ANNOT (Supplementary Figure 1).

The ARGs identified by ResFinder (n=141) were distributed in 72 ruminal microbial genomes, with greater abundance among members of the *Proteobacteria* phylum (27.3%), followed by *Bacteroidetes* (26.0%), *Actinobacteria* (21.9%) and *Firmicutes* (14.7%). ResFinder was particularly useful in identifying tetracycline resistance genes (61 genes or 43.3% of the total). Genes encoding resistance to glycopeptides and MLS (macrolide, lincosamine and streptogramin) were also predicted in the genomes of ruminal bacteria as well as genes for eight other classes of antibiotics, but their abundance was much lower compared to the tetracycline genes (Supplementary Figure 2).

When the ruminal microbial genomes were aligned against the ARG-ANNOT database using BLASTn, a total of 754 resistance genes were identified in 93 genomes (21.4%). The phylum *Bacteroidetes* had the highest proportion of genomes harboring resistance genes (44.0%),

followed by *Proteobacteria* (31.8%), *Actinobacteria* (25.0%) and *Firmicutes* (17.9%). Most ARGs predicted in ruminal microbial genomes using ARG-ANNOT were related with resistance to beta-lactams (59.2%), followed by tetracycline resistance genes (17.2%), and resistance to glycopeptides (10.1%) (Supplementary Figure 2).

Resfams identified the highest number of ARGs in the ruminal microbial genomes (3148 genes in 430 genomes). It should be noted, however, that the majority of these genes (1749 genes or 55.6% of the total) encoded putative ABC efflux pumps, which might not be directly associated with resistance to antibiotics. The second most abundant group of ARGs detected by Resfams (412 genes, 13.1% of the total) was related to vancomycin resistance, followed by resistance to beta-lactams (274 genes, 8.5% of the total) and, in a smaller account, other multidrug and non-specific mechanisms of antibiotic resistance (380 genes) (Supplementary Figure 2). No ARGs were identified by Resfams, ResFinder and ARG-ANNOT in *Prevotella albensis* DSM 11370 and in *archaea* (*Methanobrevibacter boviskoreani* JH, *Methanobrevibacter wolinii* SH, *Methanomicrobium mobile* DSM 1539 and *Methanosarcina* sp. DSM 11855), except for the presence of ABC efflux pumps found in six strains of the phylum *Euryarchaeota* using Resfams.

Phylogenetic distribution of ARGs

Although ARGs were widely distributed across the genomes of ruminal bacteria (Figure 1), we observed that resistance to specific antibiotic classes were more prevalent in some bacterial taxa, especially at the family and genus level. Members of the *Proteobacteria* phylum showed a high proportion of genomes harboring resistance genes, with ARGs being detected in all genomes of the *Enterobacteriaceae* family. In the *Bacteroidetes* and *Actinobacteria* phyla, ARGs were concentrated in the genus *Bacteroides* and *Bifidobacterium*, respectively. In the *Firmicutes* phylum (which corresponded to the largest number of genomes analyzed in this study), resistance genes were detected across members of the *Lachnospiraceae* (*Lachnoclostridium*, *Clostridium* and *Blautia*) and

Veillonellaceae (*Selenomonas*, *Megamonas* and *Megasphaera*) families as well as in all strains of the genus *Enterococcus* and *Staphylococcus*. No antibiotic resistance was detected in genomes belonging to the *Spirochaetes*, *Fibrobacteres* and *Fusobacteria* phyla, but it should be noted that the number of genomes representing these taxa were much lower compared to the other phyla analyzed in this study.

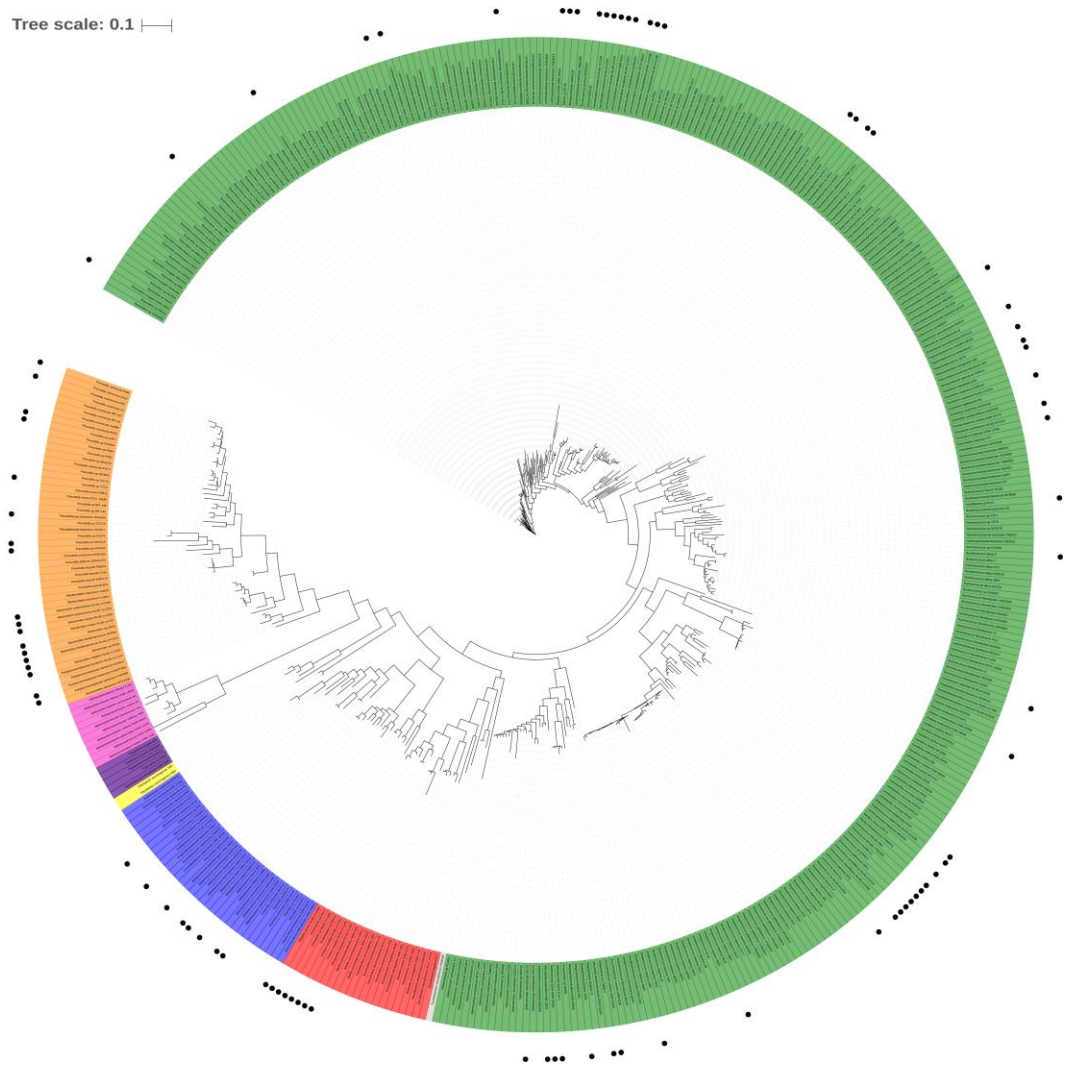

Figure 1. Phylogenetic tree of the 16S rRNA gene of ruminal microbial genomes analyzed for the presence of ARGs. Colors represent the phyla analyzed: *Actinobacteria* is shown in blue, *Firmicutes* in green, *Proteobacteria* in red, *Bacteroidetes* in orange, *Fibrobacteres* in yellow, *Fusobacteria* in gray, *Euryarchaeota* in pink and *Spirochaetes* in purple. Black circles outside the tree represent the genomes in which ARGs were identified from at least two out of the three databases (ResFinder,

Resfams and ARG-ANNOT) used in this study. The 16S rRNA gene sequences were obtained from the Hungate1000 collection and aligned using RDP Aligner. The phylogenetic tree was generated with the Maximum Likelihood method using FastTree (1000 replicates) and visualized and annotated using iTOL. Branch lengths represent the number of substituions per site.

The distribution of the ARGs in ruminal microbial genomes was represented by antibiotic class and according to the 16S rRNA gene phylogeny (Figure 2). The majority of the genomes (69.2%) showed resistance to only one class of antibiotics, with a dominance of genomes (66.6%) harboring tetracycline resistance genes. The other genomes harbored resistance genes to a minimum of two and a maximum of five antibiotic classes. Metronidazole and fosfomycin resistance were only identified in the genus *Prevotella* and in strains of *Staphylococcus epidermidis*, respectively. *Staphylococcus epidermidis* together with *Citrobacter* sp. NLAE-zl-C269 were the only species harboring resistance genes to quinolones. *Escherichia coli* PA-3 was the single genome showing the highest number of ARGs (n=5), including tetracycline resistance, beta-lactam resistance, aminoglycoside resistance, trimethoprim resistance and sulphonamide resistance. The beta-lactam resistance genes were concentrated in the family *Enterobacteriaceae*, in the genus *Bacteroides* and in two clades harboring the genera *Selenomonas*, *Staphylococcus*, *Succiniclasticum* and *Bacillus*. *Proteus mirabilis* NLAE-zl-G534 and *Proteus mirablis* NLAE-zl-G285 shared the same pattern of resistance to tetracycline and phenicol, while *Staphylococus epidermidis* AG42 and *Staphylococus epidermidis* NLAE-zl-G239 harbored highly homologous resistance genes for beta-lactams, quinolones and fosfomycin.

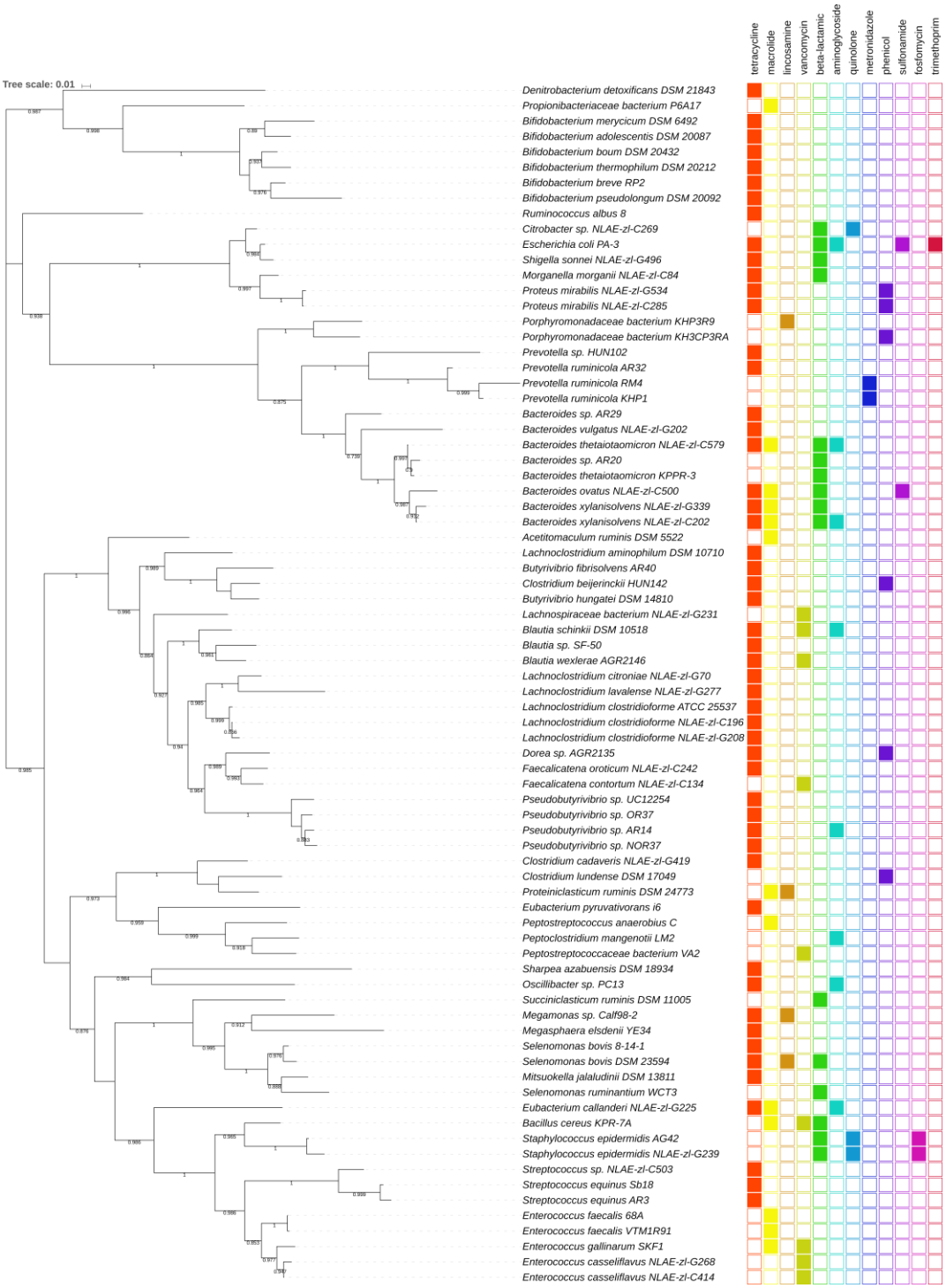

Figure 2. Phylogenetic tree of the 16S rRNA gene of ruminal microbial genomes harboring resistance genes. The distribution of the resistance genes detected by at least two out of the three computational tools used in this study (ResFinder, Resfams and ARG-ANNOT) is presented in front of the tree as filled colored boxes. The 16S rRNA gene sequences were obtained from the Hungate1000 collection and aligned using RDP Aligner. The tree was generated with the Maximum Likelihood method using

FastTree (1000 replicates) and visualized and annotated using iTOL. Only bootstrap values greater than 0.7 are shown.

Several acquired ARGs detected by ResFinder were distributed in different species, genera and phyla of ruminal bacteria (Supplementary Table 1). Tetracycline resistance genes were distributed in 54 genomes within the phyla *Actinobacteria*, *Firmicutes*, *Proteobacteria* and *Bacteroidetes* (Figure 3), with the *tet*(W) gene being shared (minimum sequence identity of 94.9% and 99% coverage) by 28 ruminal microbial genomes. Phylogenetic analysis of the tetracycline resistance genes (*tet*(W), *tet*(Q), *tet*(O)), which represented some of the most prevalent ARGs found in the ruminal microbial genomes, revealed that *tet*(W) was highly conserved across different taxa of ruminal bacteria (Supplementary Figures 3, 4 and 5). Conversely, some ARGs were restricted to a few bacterial species belonging to the same phylum, such as vancomycin resistance in *Enterococcus* spp. and in *Blautia schinkii*. Fluoroquinolone and fosfomycin resistance, conferred by *norA* and *fosA* or *fosB*, respectively, were also detected only in members of the *Firmicutes* phylum.

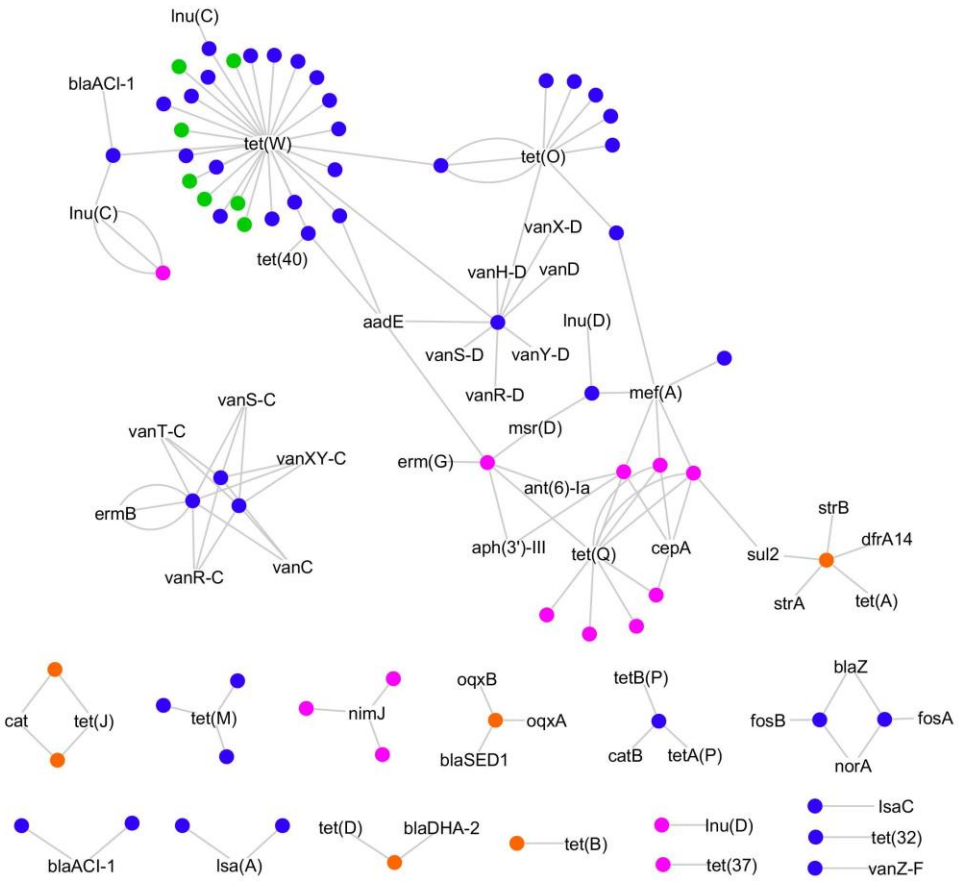

Figure 3. Network of acquired ARGs identified using ResFinder and shared among genomes of ruminal bacteria. The network was constructed using Cytoscape 3.2.1. Colored dots represent different genomes of ruminal bacteria sharing a commom ARG. Genomes belonging to the phylum *Actinobacteria* are shown in green, *Bacteroidetes* in pink, *Firmicutes* in blue and *Proteobacteria* in orange.

ARGs genetic context

The genetic locus of the *tet*(W) genes as well as their flanking regions were analyzed to investigate their mobility potential. For this, the scaffolds harboring the *tet*(W) genes found in 28 ruminal microbial genomes were analyzed and putative ORFs located up to 2000 bp upstream and 2000 bp dowsntream of the *tet*(W) gene were identified. Genes encoding a putative methyltransferase protein were identified adjacent to or located immediately upstream or downstream of the *tet*(W) gene in 18 genomes. From these, the protein under the accession number

D1PK82 in the Uniprot database, was the most prevalent one, with 98.9% to 100% sequence identity across the genomes of ruminal bacteria. In seven of these genomes, the Maff-2 protein, which is known to be involved in bacterial resistance to tetracycline, was also found flanking the *tet*(W) gene. Transposon proteins (e.g. TnpW, TnpV, Tnp, TnpX, ISPsy9) were identified flanking the *tet*(W) gene in 10 ruminal microbial genomes, and 22 out of the 28 genomes harboring *tet*(W) genes also had genes involved in plasmid transfer during bacterial conjugation, such as relaxases, plasmid mobilization proteins (e.g. MobC) and conjugation proteins (e.g. TraE, TraG/TraD). Additionally, phage proteins (e.g. PhiRv2) were detected in the flanking regions of the *tet*(W) genes in 10 ruminal microbial genomes (Supplementary Figure 6). Nonetheless, when we searched the scaffolds carrying the *tet*(W) genes for complete mobile elements (plasmids, phages and transposons) using specific softwares (PlasmidFinder, Phaster and ISfinder), no positive matches were obtained, indicating that these mechanisms alone cannot explain the widespread dissemination of the tetracycline resistance.

However, when we used ICEberg to screen the ruminal microbial genomes for ICEs in the scaffolds containing *tet*(W) genes, positive matches for the TnB1230 element were found in 27 genomes. Curiously, only a region of approximately 2400 pb (corresponding to the *tet*(W) genes and Maff-2 protein) showed high sequence identity and coverage with the TnB1230, an element with 11.849 pb in size. A more detailed analysis of the regions flanking the *tet*(W) gene in the 28 genomes revealed that at least seven genomes contained the complete machinery required for a functional ICE, suggesting that this element might mediate the spread of tetracycline genes in the rumen. Among the proteins identified in the ICEs of ruminal bacteria we found mobilization proteins (e.g. MobC), conjugation proteins (e.g. TraE), secretion proteins (e.g. VirD4), proteins related to cellular replication (e.g. DnaC, RepA, DnaI, NatR, YiaG, σ70) and recombinases (e.g. TnpX), in addition to other accessory proteins commonly found associated with these mobile genetic elements (e.g. methyltransferases, transposons and phage-related proteins), as indicated for the genome of *Blautia schinkii* 10518 (Figure 4). The hypothesis that a

novel ICE (named here as ICE_*RbtetW_07*) could be mediating tetracycline resistance in the rumen was reinforced by the linear arrangements (reversed in three genomes) of the gene loci on the chromosomes and the presence of highly conserved elements in the flanking regions of *tet*(W) genes detected in the genomes of ruminal bacteria (Figure 5).

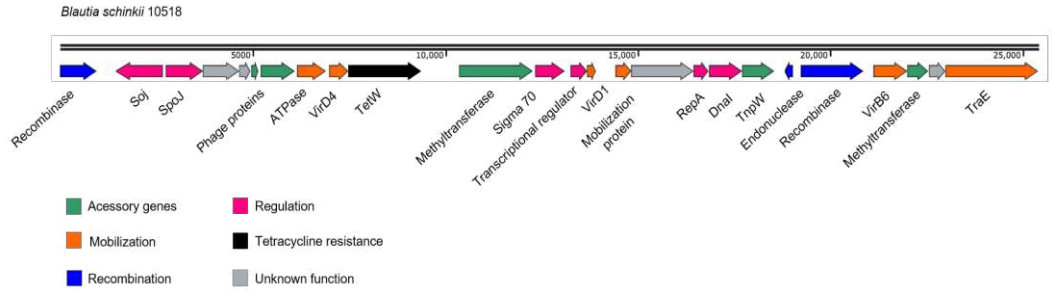

Figure 4. Schematic representation of the ICE_*RbtetW_07* structure carrying the *tet*(W) gene in *Blautia schinkii* 10518 constructed using the software SnapGene 2.3.2. The genes are represented by arrows classified and colored in modules of ICE according to their function. The essential modules of the ICE machinery are represented in orange, blue and pink, for mobilization, recombination and regulation genes, respectively.

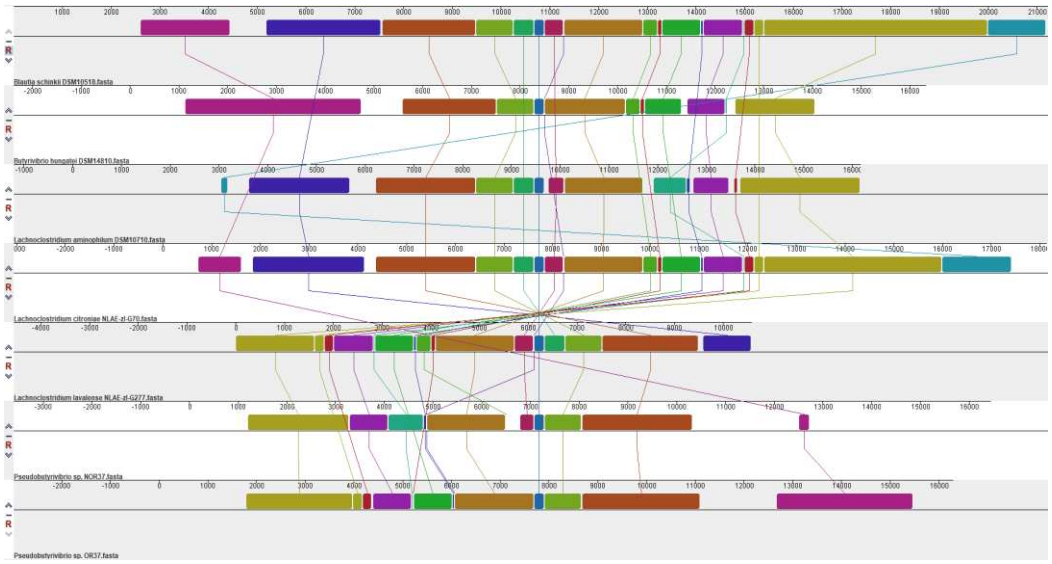

Figure 5. Alignment of the ICE_*RbtetW_07* sequences found in the scaffolds containing *tet*(W) genes in 7 ruminal microbial genomes. The sequences were aligned using progressiveMAUVE in MAUVE software.

Lines connecting blocks with identical colors represent the aligned regions and show synteny or gene rearrangements. The *tet*(W) sequence is represented in blue in the center of the figure.

Selective pressure analysis of tetracycline resistance genes

The tetracycline resistance genes *tet*(W), *tet*(Q) and *tet*(O) were highly abundant and widely distributed in the genomes of ruminal bacteria. To investigate if these genes are under selective pressure, the number of non-synonymous per synonymous substitutions (d_N/d_S ratio) was calculated to determine the codon substitution rate. Our analysis confirmed the presence of positive selection pressure in the *tet*(W) gene, with a $d_N/d_S > 1$ for 36 out of the 639 amino acid residues in the protein (Figure 6a). This positive selection sites was mainly clustered in three regions of the *tet*(W) protein, between the amino acids at positions 141 to 265, 310 to 341 and 600 to 639, with an average d_N/d_S ratio per site of 1.9, 2.6 and 3.1, respectively. For the *tet*(Q) and *tet*(O) genes, our analysis indicated 25 and 17 amino acid sites with positive selection pressure, respectively (Figure 6b and c). The overall d_N/d_S ratio calculated for these genes was less than 1, and the Likelihood Ratio Test Data (LRT) for the substitution models did not present statistically significant difference at 95% confidence level, indicating the absence of positive selection pressure for *tet*(O) and *tet*(Q).

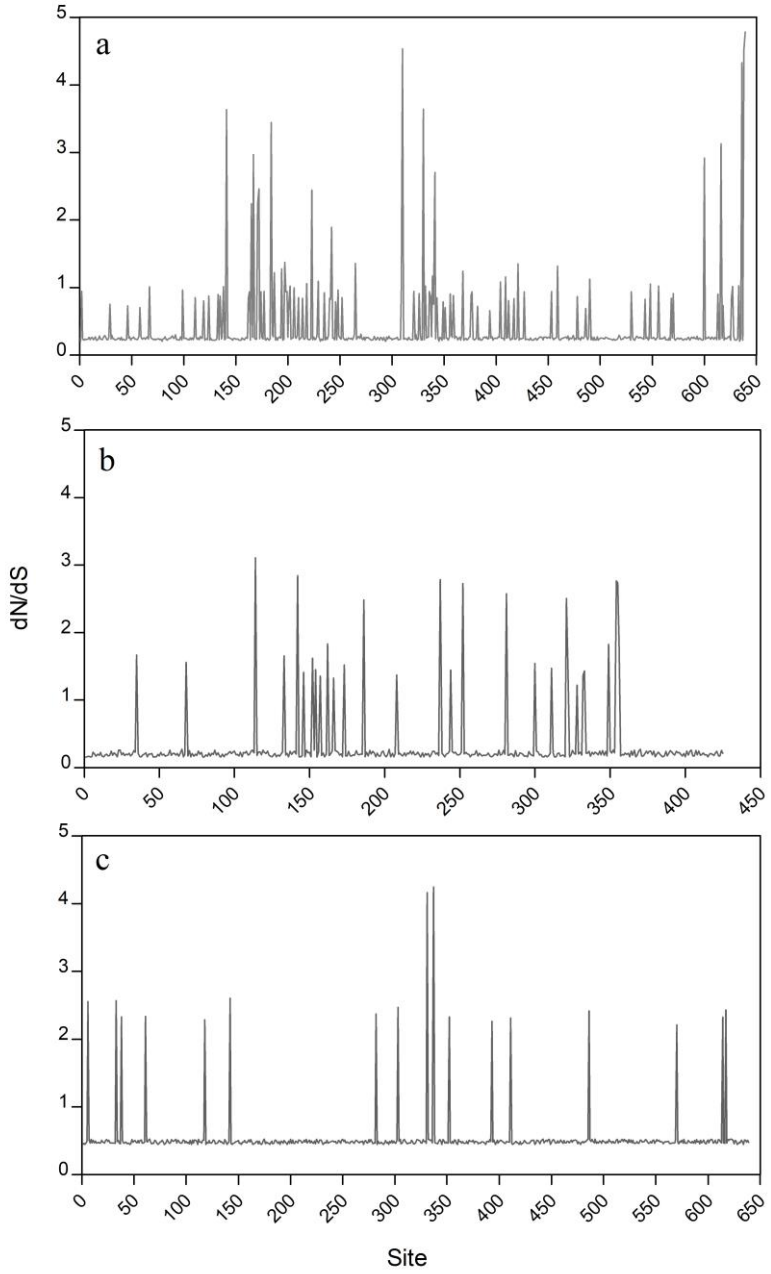

Figure 6. d_N/d_S ratio by site in tetracycline resistance genes from ruminal microbial genomes using JCoDA. The d_N/d_S ratio was calculated for the *tet*(W) (a), *tet*(Q) (b), and *tet*(O) genes (c). The line represents the score of d_N/d_S by site. The phylogenetic tree generated by JCoDA for d_N/d_S analysis was reconstructed using the aligned protein sequences of the ARGs and Maximum Likelihood approach with 100 replicates.

Expression of ARGs in metatranscriptomes

To confirm if the detected ARGs in ruminal microbial genomes were present and active in the GIT of ruminants and humans, we aligned sequences of resistance genes to the five most abundant antibiotic classes (aminoglycoside, beta-lactam, macrolide, tetracycline and vancomycin) to the metranscriptome datasets of ruminal contents of dairy and beef cattle and fecal samples of a human subject. At least one resistance gene from each antibiotic class was expressed in one or more of these metatranscriptomes. Genes conferring resistance to aminoglycosides were expressed only in the human dataset. The other classes of antibiotics were expressed both in ruminants and humans, although individual genes varied in their expression level between different datasets and some genes were not expressed in all metatranscriptomes.

In general, tetracycline resistance genes presented the highest level of expression while vancomycin resistance was the least expressed. There was also greater similarity between the genes being expressed in the metatranscriptome of dairy and beef cattle, with higher RPKM (reads per kilobase in a gene per million mappable reads) values obtained in the dairy cow dataset (Table 1). It should be noted that many ARGs originally detected in the ruminal microbial genomes were also expressed in the human gut metatranscriptome, especially genes encoding resistance to aminoglycosides and tetracycline. Some of the ARGs identified in the ruminal microbial genomes were not expressed in any of the metatranscriptome datasets investigated in this study, including the aminoglycoside *strA*, *strB*, beta-lactam *blaSED1*, *blaDHA*-2, *blaZ*, macrolide *erm*(G), *lsa*(A), *lsaC*, tetracycline *tetA*(P), *tetB*(P), *tet*(D), *tet*(J), *tet*(M), and vancomycin *vanC*, *vanD*, *vanH*-D, *vanR*-D, *vanT*-C, *vanX*-D, *vanXY*-C, *vanZ*-F resistance genes. Although we could expect a difference in expression of ARGs if other metatranscriptome datasets were used and the ARG profiles in these particular datasets might not be representative of beef cattle, dairy cattle and human subjects in general, our results still confirm that several ARGs detected in ruminal microbial genomes were also active in the GIT microbiota of humans and ruminants.

Table 1. Expression levels (RPKM* values) of ARGs in metatranscriptomes of beef and dairy cattle and the human gut. The five main classes of ARGs identified in ruminal microbial genomes were analyzed using Bowtie2.

Antibiotic class or group	Genes	Datasets		
		human gut (RPKM)	beef rumen (RPKM)	dairy rumen (RPKM)
Aminoglycoside	*aadE*	+++		
	ant(6)-Ia	++		
	aph(3')-III	+++		
Beta-lactam	*cepA*	+++		
	blaACI-1		+	+
Macrolide	*ermB*	+++		
	mef(A)		+	++
Tetracycline	*tet*(32)	+++	+	+
	tet(40)	+++	+	++
	tet(A)	+++		
	tet(B)	++		
	tet(O)	++	+	+
	tet(Q)	++++	++	+++
	tet(W)	++++	++	+++
	tet(37)	++		+
Vancomycin	*vanR*-C			+
	vanS-C			+
	vanS-D	++		
	vanY-D		+	

+: 0.01-0.1; ++: 0.1-1; +++: 1-10; ++++: 10-164; *RPKM = reads per kilobase million

In vitro analysis of antibiotic resistance in ruminal bacteria

To validate the resistance phenotype that was computationally predicted in the genomes of ruminal bacteria, twenty-six pure cultures of ruminal bacteria matching the genomes analyzed *in silico* were selected for further *in vitro* characterization. Our *in silico* analysis identified 57 resistance genotypes in the ruminal bacteria selected for our *in vitro* assays. According to the minimum inhibitory concentration (MIC values) determined using the Epsilometer test (*E*-test) method, 18 resistance phenotypes were confirmed *in vitro*, which included, in some cases, resistance to more than one antibiotic in the same class (Table 2).

Comparing the approaches used to predict and confirm the antibiotic resistance phenotype, we observed that eight ARGs (14.0% of the total) were detected/confirmed both *in silico* and *in vitro* by all approaches used in this study (Supplementary Figure 7). ARG-ANNOT predicted 14 ARGs in the 26 cultures of ruminal bacteria tested *in vitro*. From this, 10 resistance phenotypes (71.4%) were confirmed in the *in vitro* assays. ResFinder and Resfams predicted 14 and 53 ARGs in our pure cultures and resistance to 9 (64.3%) and 17 (32.1%) of these antibiotics were confirmed phenotypically (Supplementary Figure 7). Therefore, integrating the analysis of ARGs in the 26 genomes and their respective cultures, it was observed that ResFinder, ARG-ANNOT and the *in vitro* analysis agreed in most part of the results, while Resfams, on the other hand, predicted several resistance mechanisms that were not confirmed by the other methodologies.

Table 2. Susceptibility of ruminal bacteria to different classes of antibiotics. Antimicrobial resistance was evaluated by determining the MIC (Minimum Inhibitory Concentration) using the Epsilometer test (E-test) method. Results were interpreted according to the established EUCAST breakpoints.

Bacteria	Strains	Beta-lactam		Aminoglycoside		Tetracycline[1]	Vancomycin	Chloramphenicol	Macrolide[1]		
		Ampicilin	Amoxicillin	Gentamicin[3]	Streptomycin*				Erythromycin	Azithromycin	Clindamycin
Acetitomaculum ruminis	5522						S				S
Bifidobacterium adolescentis	20087	S	S			R					
Bifidobacterium boum	20432	S	S			R					
Bifidobacterium merycicum	6492			S	4	S					
Bifidobacterium pseudolongum	20092			R	64	R					
Bifidobacterium thermophilum	20212					R					
Blautia schinkii	10518	S	S	S	12	R	R				
Butyrivibrio fibrisolvens	3071			S	4		S				
Butyrivibrio proteoclasticum	B316			S	4		S				
Butyrivibrio sp.	M55	S	S				S				
Clostridium aerotolerans	5434	S	S	S	8	S	S	S			
Clostridium intestinale	6191	S	S			S	R				
Clostridium lundense	17049	S	S				R	S	R	S	
Clostridium polysaccharolyticum	1801						R				

(continue)

(continuation)

Bacteria	Strains	Beta-lactam		Aminoglycoside		Tetracycline[1]	Vancomycin	Chloramphenicol	Macrolide[1]		
		Ampicilin	Amoxicillin	Gentamicin[3]	Streptomycin*				Erythromycin	Azithromycin	Clindamycin
Lachnoclostridium aminophilum	10710	S	S			R	S				
Lachnospira multiparus	D15d	S	S								
Lactobacillus ruminis	20403						R				
Megasphaera elsdenii	T81	S	S								
Mitsuokella jalalundinii	13811	R	R			R					
Olsenella umbonata	22619	S	S								
Prauserella rugosa	43194	S	S						R	R	
Proteiniclasticum ruminis	24773	S	S	I	8		S[2]		S	S	S
Ruminococcus flavefaciens	Fd1						S				
Selenomonas ruminantium	2872	R	R								
Sharpea azabuenensis	18934	S	S			R	S				
Sharpea azabuenensis	20406	S	S				S				

*There are no interpretative standards for streptomycin and the MIC values (µg/ml) are reported; [1]Breakpoint value (BV) for *Streptococcus* sp.; [2]BV for Gram positive bacteria; [3]BV for *Enterobacteriaceae*. R = resistant; S = susceptible; I = intermediate; White space: not tested (only resistance phenotypes predicted *in silico* was tested *in vitro*).

In addition, tetracycline resistance was detected simultaneously by at least three approaches in 69% of the genomes and their respective cultures, providing further evidence that tetracycline resistance is widespread in the rumen ecosystem. In the case of the other antibiotic classes, resistance was often predicted by only one computational tool and, in some cases, was also confirmed by our *in vitro* assays, as the vancomycin resistance in the species of *Clostridium* and *Lachnoclostridium* and in *Lactobacillus ruminis* (Figure 7). We also confirmed *in vitro* the resistance of *Mitsuokella jalalundinii* and *Selenomonas ruminantium* to beta-lactams, *Bifidobacterium pseudolongum* to aminoglycoside as well as macrolide resistance in *Clostridium lundense* and *Prauserella rugosa*.

Figure 7. Schematic representation of the agreement between *in silico* and *in vitro* analysis of the ARGs in the 26 ruminal bacteria analyzed by the four approaches. Bar color represent the approach that predicted/detected the respective antibiotic resistance in each bacteria: in yellow Resfams, in green ResFinder, in orange ARG-ANNOT and in red the *in vitro* analysis.

Discussion

The hypothesis that livestock animals represent a reservoir of ARGs is not new and previous studies have demonstrated that bacteria isolated from fecal samples of monogastrics and ruminants harbor genetic elements that can confer resistance to clinically used antibiotics[21]. However, less attention has been given to the occurrence of ARGs in the rumen ecosystem and their potential to be transferred to commensal and pathogenic bacteria. Microorganisms colonizing the rumen also disseminate to the environment through animal saliva during rumination and due to the flow of rumen microbial biomass to the omasum and abomasum to the distal parts of the GIT, being released through fecal discharge into the soil[22].

In this work, we investigated the occurrence and distribution of ARGs in hundreds of ruminal microbial genomes made available through the Hungate1000 project[17]. Our results indicate that ARGs are widely distributed among ruminal bacteria, with genes conferring resistance to tetracycline being frequently detected in the genomes of several species. The regions flanking the tetracycline resistance genes showed a conserved pattern in an apparently novel integrative and conjugative element (ICE), suggesting dissemination of antibiotic resistance through horizontal gene transfer in the rumen microbiome. In addition, we show evidence of positive selective pressure on the *tet*(W) gene, which may contribute to the maintenance and conservation of tetracycline resistance genes among ruminal bacteria.

Tetracyclines are broad-spectrum antibiotics that inhibit protein synthesis in susceptible bacteria[23] and are often used in clinical settings to fight bacterial infections in humans and to promote the growth of food-producing animals, improving feed efficiency and animal performance[24]. Analysis of metagenomic sequencing data has shown that genes conferring resistance to tetracycline are abundant in the human gut, a feature that appears to correlate with the use of antibiotics in food animals[25]. Tetracycline resistance is usually mediated by efflux pumps (e.g. *tet*(A), *tetA*(P), *tet*(B), *tet*(J), *tet*(40)), protection of the ribosomal

binding site (e.g. *tet*(M), *tet*(O), *tet*(W), *tet*(Q), or *tet*(32)) or enzymatic drug modification (e.g *tet*(X), *tet*(34), *tet*(37))[26]. Among these, the gene *tet*(M) is most frequently associated with tetracycline resistance in human bacteria[26], whereas *tet*(W), *tet*(Q) and *tet*(O) are commonly detected in fresh feces of cattle, being designated as the “core resistome” of these animals[27]. In our study, *tet*(W), *tet*(Q) and *tet*(O) were also the main tetracycline resistance genes identified by ResFinder, but *tet*(W) was the most abundant gene, being distributed in 28 genomes of ruminal bacteria with high similarity of nucleotide sequences. ResFinder is an online platform that uses whole genome sequencing data to identify acquired AMR genes in bacteria[18]. An earlier study demonstrated the presence of the *tet*(W) gene among members of the rumen microbiome, being carried by distinct genera such as *Selenomonas*, *Mitsuokella* and *Butyrivibrio*. The high sequence identity of the *tet*(W) gene among these genera was explained by horizontal gene transfer events, but the elements mediating this transference were not well described[28]. Therefore, in this study, the *tet*(W) gene identified by ResFinder was selected for further analyses aiming to investigate evidences of horizontal transfer among ruminal bacteria and the potential mechanisms mediating this transfer.

Tetracycline resistance genes are often localized within transposons and several of these genetic elements have been reported. In earlier work, authors described the composite transposon Tn2440, which carries a tetracycline resistance region, as the first identified transposon with direct repeats (IS160) as flanking regions[29]. Additionally, it was reported the presence of *tet*(W) gene in the conjugative transposon element TnB1230 of *Butyrivibrio fibrisolvens* isolated from the bovine rumen[30]. Our results showed that the some of the *tet*(W) genes detected in ruminal microbial genomes harbored in their flanking regions a protein (Maff-2) with high identity to the TnB1230 element. Maff-2 is a transmembrane protein that is known to flank direct repeats of transposable elements carrying tetracycline resistance genes in TnB1230[31]. Several ruminal microbial genomes also had a putative protein with a methyltransferase domain flanking the *tet*(W) gene. Methylases are commonly found in mobile genetic elements (MGEs), such as ICEs, and

are often involved in the protection of nucleotide sequences against the host restriction-modification system, which degrades non-methylated DNA recognized as foreign sequences by the host[32].

Furthermore, our analyses suggest that the *tet*(W) gene of several species of ruminal bacteria are located on an ICE with a unique modular pattern, not previously reported for tetracycline resistance. ICEs are characterized by the presence of the essential conjugation, recombination and regulation modules[33]. The conjugation machinery often contains relaxases and secretion systems to mediate the transfer of DNA between donor and recipient cells. The recombination module usually contains genes encoding integrases, excisionases and recombinases, while genes encoding proteins involved in DNA transcription are found in the regulation module. Moreover, several accessory genes including methyltransferases, transposons, phages and plasmid related proteins, in addition to the ARGs, can be present in an ICE structure[33]. At least one essential gene from each module and several accessory genes were detected in the flanking region of the *tet*(W) identified in seven ruminal microbial genomes in our study, suggesting that ICEs could be mediating the horizontal transfer of the tetracycline resistance genes between ruminal bacteria. Our analysis also demonstrated that the mobilization module, which contained the MobC and VirD4 protein is conserved in these elements, as well as the regulation module with the presence of DnaI/DnaC, RepA and a transcriptional regulator. Furthermore, accessory genes encoding for methyltransferases were present in all ICEs identified in the ruminal microbial genomes. The transfer of ICEs between different species of bacteria is not a common event, and our analysis indicated that this might be the case among the genomes of ruminal bacteria. If this is indeed the case, there is a potential threat to the transfer of these genetic elements to bacterial pathogens in the GIT of ruminants and in the environment.

In addition, our results demonstrate a positive selective pressure on the *tet*(W) gene. The strong exposure of livestock to tetracycline, the most commonly used antibiotic class for animals worldwide[34], may explain the dominance of tetracycline resistance genes in animal gut microbiomes. In 2015, tetracycline was the best-selling antibiotic for agricultural use,

accounting for 44% of the total sales of clinically relevant antibiotics being used in livestock animals[7]. The acquisition of ARGs by transfer of MGEs appears to increase under selective pressure[35]. More recently, it was also observed that the abundance and diversity of ARGs and MGEs increased in the intestinal microbiota of mice exposed to 10 g/L of tetracycline for two weeks[36].

We also demonstrate substantial differences in the number and classes of ARGs detected using different computational tools. ARG-ANNOT detected a high abundance of beta-lactam resistance genes in the ruminal microbial genomes. Although these findings may be due to an overrepresentation of beta-lactam resistance genes in the ARG-ANNOT database, the predominance of beta-lactam resistance in the ruminal microbial genomes could also be related to the intensive use of beta-lactams in livestock production systems. This idea is supported by recent reports showing that penicillin represent the second class of human clinically-related antibiotic most commercialized for agricultural applications[7]. In addition, the differences found in the profiles of ARGs detected in ruminal bacteria by Resfinder, Resfams and ARG-ANNOT are mainly due to their distinct ARG databases and to the use of different computational approaches (BLASTn for Resfinder and ARG-ANNOT and Markov chain for Resfams) to identify the resistance genes.

Furthermore, our *in silico* analysis showed an abundance of vancomycin resistance genes in the ruminal microbial genomes, especially when genome mining was performed using Resfams. A complete operon for vancomycin resistance was found in the genomes of *Enterococcus casseliflavus* and *Enterococcus gallinarum*, which have been associated with infections in humans, such as bacteremia and meningitis[37,38]. Vancomycin is one of the “last-option” antibiotic used to treat infections caused by Gram-positive bacteria[39], being used as a first-line medicine in the treatment of moderate and severe *Clostridium difficile* infections in patients diagnosed with pseudomembranous colitis[40]. Although vancomycin-resistance is not considered widespread in *Clostridium*[41], several resistant strains have been isolated from humans. For example, a US-based national sentinel surveillance study detected resistance to

vancomycin, based on the EUCAST breakpoint, in 17.9% of the *C. difficile* isolates obtained from patients with diarrhea[42]. Additionaly, all *Clostridium innocum* isolates (n=24) obtained from extra-intestinal infections sites in humans were resistant to vancomycin[43]. Moreover, the transference of a vancomycin transposon (Tn1549) from *Clostridium symbiosum* to *Enterococcus faecium* and *Enterococcus faecalis* was demonstrated in the digestive tract of gnotobiotic mice in *in vivo* experiments[44], thus suggesting a potential threat to human health. Nonetheless, there is a lack of information about vancomycin resistance in ruminal species of *Clostridium* as well as in *Blautia*, where our *in silico* analysis predicted a complete operon encoding vancomycin-resistance and the phenotype was confirmed *in vitro*.

The fact that vancomycin resistance genes were abundant in ruminal microbial genomes is a matter of concern, since vancomycin should have limited use for therapeutic purposes in animals and the use of vancomycin analogues as growth promoter in livestock has been restricted[39]. Antibacterial molecules structurally similar to vancomycin, such as avoparcin, were used for almost 30 years as growth promoters in farm animals until they were banned in the late 1990's, together with other glycopeptide antibiotics, by the European Union[45]. Avoparcin is known to select for vancomycin resistant enterococci (VRE) in food-producing animals and previous studies suggested that VRE from livestock could potentially transfer resistance to human pathogens[46].

Our results also demonstrated that members of the *Proteobacteria* phylum can harbor multiple ARGs, as exemplified by the genome of *E. coli* PA-3, which contains five resistance genes conferring resistance to five distinct antibiotics. The enrichment of ARGs appears to be a common feature in the mobile resistome of *Proteobacteria* from the animal and human gut microbiome[47]. This may be associated with the frequent use of antibiotics targeting *Enterobacteriaceae* to control infections in the respiratory, urinary and GIT of humans and livestock, thus selecting for resistant strains[13].

To evaluate if these ARGs are expressed in the rumen and human GIT microbiota, we performed computational analysis to map sequences

of the genes encoding resistance to different metatranscriptome datasets. Our *in silico* analysis indicated that the expression of genes encoding resistance to tetracycline, vancomycin, macrolides, beta-lactams and aminoglycosides varied between the rumen of dairy and beef cattle and the human gut. Nonetheless, some genes were being expressed in all datasets, showing evidence of an active role and co-existence of these genes in different environments. This observation is also interesting considering that our *in vitro* assays confirmed only 31.6% of the resistance phenotype predicted *in silico*. This suggests that the standardized conditions used to test the bacterial phenotypes might not mimic the growth conditions of the ruminal bacteria in their natural habitat, which could influence the susceptibility to the tested antibiotics.

Taken together, our results demonstrate the prevalence and widespread distribution of ARGs, especially tetracycline resistance genes, among ruminal bacteria. Our findings demonstrate that ARGs, in particular the genes for tetracycline resistance, are prevalent among the ruminal bacteria and subjected to positive selection pressure. Moreover, analysis of the flanking regions of the *tet*(W) genes provided evidence that this gene can be disseminated by horizontal transfer. Given the fact that the rumen ecosystem also harbor a highly dense and diverse microbiota that maintain complex ecological associations, and the capacity of microorganisms to exchange DNA through direct (conjugation) and indirect mechanisms (transformation, transduction), the GIT of ruminants should be considered as a relevant source of genes for antibiotic resistance. The evidence of a new ICE (ICE_*RbtetW_07*) carrying the *tet*(W) gene emphasizes this idea and presents a risk for both animal and human health, due to the capacity of these genetic elements to disseminate in the environment. This hypothesis is also supported by the observation of non-phylogenetically related bacteria harboring the same resistance genes, and the expression of the same ARGs in both beef and dairy cattle and in human datasets. Additionally, our results highlight the importance of using multiple computational tools to search for ARGs in genomic data and demonstrate the relevance of developing adequate *in vitro* assays to confirm the resistance phenotypes.

Material and Methods

Collection of genomic data from ruminal microorganisms and identification of ARGs

The sequence files of 435 microbial genomes (425 bacteria and 10 archaea), from the Hungate1000 project, were downloaded in FASTA format from the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/genome) and the JGI (Joint Genome Institute, http://genome.jgi.doe.gov) websites (Supplementary Table 2). Putative ARGs in the bacterial and archaea genomes were searched for and screened on the ResFinder v2.1 database (https://cge.cbs.dtu.dk/services/ResFinder/)[18].

We tested all antibiotic classes available for analysis of acquired ARGs in the ResFinder database, which included aminoglycosides, beta-lactams, colistin, fluoroquinolones, fosfomycin, fusidic acid, glycopeptides, MLS (macrolide, lincosamine and streptogramin), nitroimidazole, oxazolidone, phenicol, rifampicin, sulphonamide, tetracycline and trimethoprim. For homology-based screening, the minimum gene identity and sequence length were set to 70% and 60% respectively in relation to the reference resistance gene.

The software Resfams v1.2 was used to search for conserved structural domains of antibiotic resistance function in protein sequences of ruminal bacteria[20]. The protein sequences of the analyzed genomes were downloaded in FASTA format from the NCBI or JGI websites and when these sequences were not found, the online interface Prodigal v1.2 (http://compbio.ornl.gov/prodigal/server.html) was used to convert nucleotide to protein sequences using the Standard Bacteria/Archaea translation table code.

Genomes of ruminal microorganisms were also analyzed using the ARG-ANNOT database (http://en.mediterranee-infection.com/article.php?laref=283&titre=arg-annot-)[19]. Sequences of the ARGs in the ARG-ANNOT database (ARG-ANNOT Nt V3 March 2017) were used for similarity analysis against the ruminal microbial genomes

using BLASTn[48] on the Galaxy platform[49]. The alignment parameters were minimum sequence identity of 70%, sequence length cut-off of 60% and *E*-value < 10^{-6}.

Phylogenetic analysis and distribution of ARGs in ruminal microbial genomes

To evaluate if the ARGs grouped according to the evolutionary history of the microbial species, phylogenetic trees were reconstructed using the 16S rRNA gene sequences from the genomes analyzed in this study. The sequences encoding the 16S rRNA gene were obtained from the Hungate1000 collection[17]. Next, all the sequences were organized in a single txt file and aligned using the RDP Release 11.5 aligner in the Ribosomal Database Project website (RDP, https://rdp.cme.msu.edu/)[50]. FastTree v2.1 (http://www.microbesonline.org/fasttree/)[51] was used to infer Approximately-Maximum-Likelihood phylogenetic trees using default settings. The generated output file (.tree) was visualized and annotated on the Interactive Tree of Life (iTOL) interface v4 (https://iTOL.embl.de/)[52].

To evaluate sequence conservation of the ARGs that were identified *in silico* as described above, the sequences of the most frequent genes conferring resistance were extracted from the ruminal microbial genomes and aligned as described below. To extract the sequences of the resistance genes, all genomes were annotated by Prokka v1.12[53] using Galaxy platform (minimum contig size of 200 and *E*-value < 10^{-6}). To obtain the location of the resistance genes in the bacterial genomes, the ResFinder v2.1 database was aligned against the annotated genomes using the BLASTn tool on the Galaxy platform. Minimum query coverage and sequence identity were 60% and 70%, respectively, with a threshold *E*-value < 10^{-6}. The sequence of the resistance genes identified in the previous alignment were extracted using the SAMtools (http://SAMtools.sourceforge.net/) software v1.9[54] and the most abundant ARGs were aligned using MUSCLE version 3.8.31[55]. The phylogenetic reconstruction was based on the Maximum Likelihood method using

FastTree v2.1 and iTOL v4 graphical interface was used to represent the phylogenetic trees, as described above.

Genetic context of ARGs

To assess potential mechanisms of ARG mobility, the genetic elements flanking the resistance genes in a scaffold were investigated using the software Artemis[56]. When these flanking elements were annotated as "hypothetical protein", sequence function was predicted on the UNIPROT website (http://www.uniprot.org/)[57], using BLASTn, UniProtKB as the target database and default settings. Putative mobile genetic elements flanking the resistance genes were searched using different computational tools. ISfinder (https://www-is.biotoul.fr/)[58] was used to screen for transposons and/or integrons, and phage sequences were confirmed using PHASTER (http://phaster.ca/)[59]. The genomes that harbored proteins with predicted plasmid functions near the resistance genes were further analyzed against the *Enterobacteriaceae* and Gram-positive database of PlasmidFinder 2.0 (https://cge.cbs.dtu.dk/services/PlasmidFinder/)[60], using a threshold for minimum sequence identity of 70% and minimum coverage of 60%. Finally, ICEberg 2.0 (http://db-mml.sjtu.edu.cn/ICEberg/)[61] was used to identify ARGs carried by integrative and conjugative elements (ICE). Scaffolds harboring selected ARGs were used as query sequences (FASTA format) in the WU-BLAST2 search tool in ICEberg, using BLASTn as the search program and ICE nucleotide sequence as the sequence database. All the other parameters were kept as default settings. To evaluate the conservation of the ARGs flanking regions among the ruminal microbial genomes, the sequences were aligned using progressiveMAUVE in MAUVE software[62].

Selective pressure on ARGs in ruminal microbial genomes

Sequences of the most prevalent ARGs identified in ruminal microbial genomes were selected to evaluate if these genes are under

selection pressure. Selective pressure on the ARGs was estimated by number of non-synonymous and synonymous substitutions (d_N/d_S) using the software JCoDA[63]. JCoDA uses ClustalW to align the nucleotide sequences and Phylogenetic Analysis using Maximum Likelihood (PALM) to calculate the d_N/d_S ratio. Analyses were performed using nucleotide sequences in FASTA format as input sequences and the universal (default) genetic translation code. The alignment option was set to ClustalW and phylogenetic trees were reconstructed by the Maximum Likelihood method with 100 replicates using protein sequences in the Phylip-sequential format. The d_N/d_S was calculated by site (amino acid) using the Bayes Empirical Bayes (BEB) model, which takes into account the uncertainties of model parameters, avoiding the generation of false positives[63].

Expression of ARGs in metatranscriptomes

The metatranscriptome datasets were downloaded in FASTQ format from the NCBI Sequence Read Archive SRA, (https://www.ncbi.nlm.nih.gov/sra)[64]. Three datasets representing cattle from two distinct production systems (dairy and beef) and a human subject were selected for analysis: 1) a 5.9 Gbp metatranscriptome (Bovine_rumen_microbiome_L167_metatranscriptome) from beef cattle (*Bos taurus*) (SRR3257011)[65] 2) a 15.7 Gbp metatranscriptome (rumen metatranscriptome) obtained from a lactating Holstein dairy cow (*Bos taurus*) (SRR5805524), and 3) a 1.3Gbp metatranscriptome (Illumina RNA sequencing of transcriptome paired-end library 'Georgia Giannoukos_6421_RNAlater' containing sample X319146421_RNAlater) derived from stool samples of human subjects (SRR769401)[66]. Information about these sequences is shown in the Supplementary Table 3. FastQC software v0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) was initially applied for quality control checks on raw sequence data using a Phred quality score of 20 to filter high quality bases. However, because most

reads were discarded using this criteria, gene expression was evaluated using raw sequence data.

In this study, the most abundant group of ARGs identified in the ruminal microbial genomes was selected to verify their expression in the metatranscriptomic datasets. These genes were predicted to encode resistance to aminoglycosides (*aadE*, *ant*(6)-Ia, *aph*(3')-III, *strA*, *strB*), beta-lactams (*cepA*, *blaACI*-1, *blaSED1*, *blaDHA*-2, *blaZ*), macrolides (*ermB*, *erm*(G), *mef*(A), *Isa*(A), *IsaC*), tetracycline (*tet*(32), *tet*(40), *tet*(A), *tetA*(P), *tet*(B), *tetB*(P), *tet*(D), *tet*(J), *tet*(M), *tet*(O), *tet*(Q), *tet*(W), *tet*(37) and vancomycin (*vanC*, *vanD*, *vanH*-D, *vanR*-C, *vanR*-D, *vanS*-C, *vanS*-D, *vanT*-C, *vanX*-D, *vanXY*-C, *vanY*-D, *vanZ*-F). The Bowtie2-build tool was used to index the DNA sequences of the resistance genes, while Bowtie2[67] was employed to align these sequences to the metatranscriptomic datasets. The expression level of each gene was calculated by the number of uniquely mapped reads per kilobase in a gene per million mappable reads (RPKM)[68].

In vitro studies of antibiotic resistance in ruminal bacteria

In order to evaluate if the ARGs predicted in the ruminal microbial genomes actually conferred the predicted resistance phenotype *in vivo*, twenty six cultures of ruminal bacteria that had their genomes analyzed for the presence of ARGs were tested *in vitro* to confirm these phenotypes. Cultures stored at -80 ºC were activated twice in anaerobic Brain Heart Infusion (BHI) broth at 39 ºC for 24 h under anaerobic conditions before each experiment. For the antibiotic susceptibility test, the optical density (OD_{600nm}) of the cultures was adjusted to obtain a cell count of approximately 10^8 CFU ml^{-1} (equivalent to 0.5 McFarland scale). Subsequently, cultures were spread on the surface of anaerobic solid BHI media using swabs. *E*-test tapes (Biomérieux, Basingstoke, UK; Fannin, Dublin, Ireland) of the antibiotics to be tested were distributed on the surface of the inoculated medium and the plates were incubated anaerobically at 39 ºC for another 24 h. Only antibiotics to which resistance was predicted *in silico* were evaluated *in vitro* using pure

cultures of ruminal bacteria. The minimum inhibitory concentration (MIC) of each antibiotic was determined at the intersection of the inhibition zone with the *E*-test tapes[69]. All experiments were performed with three biological replicates. Reference values from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint table were used to interpret the MIC results[70]. In the case of a few antibiotics where breakpoint values were not available for the tested species, MIC interpretations were based on breakpoint values published for the most closely related organism.

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Author contributions

YNVS, HCM, and SAH conceived the project. YNVS and AJSM completed the laboratory work under the supervision of HCM and SAH. YNVS, MFS, and FGS analysed the data and generated figures. SAH provided the anaerobic cultures analyzed in the study. LBO assisted YNVS with the *in vitro* experiments. LBO, MFS and SAH provided valuable ideas into the project from the time of conception. YNVS and HCM wrote the manuscript with imput from all co-authors.

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Supplementary information

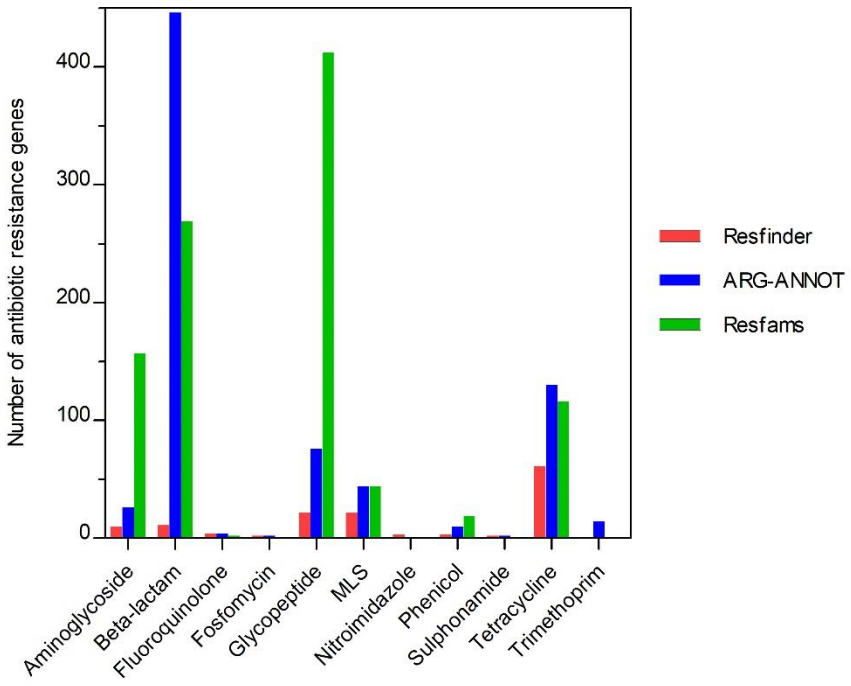


Figure 1. Number of resistance genes distributed by antibiotic class detected by ResFinder, ARG-ANNOT and Resfams in the ruminal microbial genomes analyzed in this study.

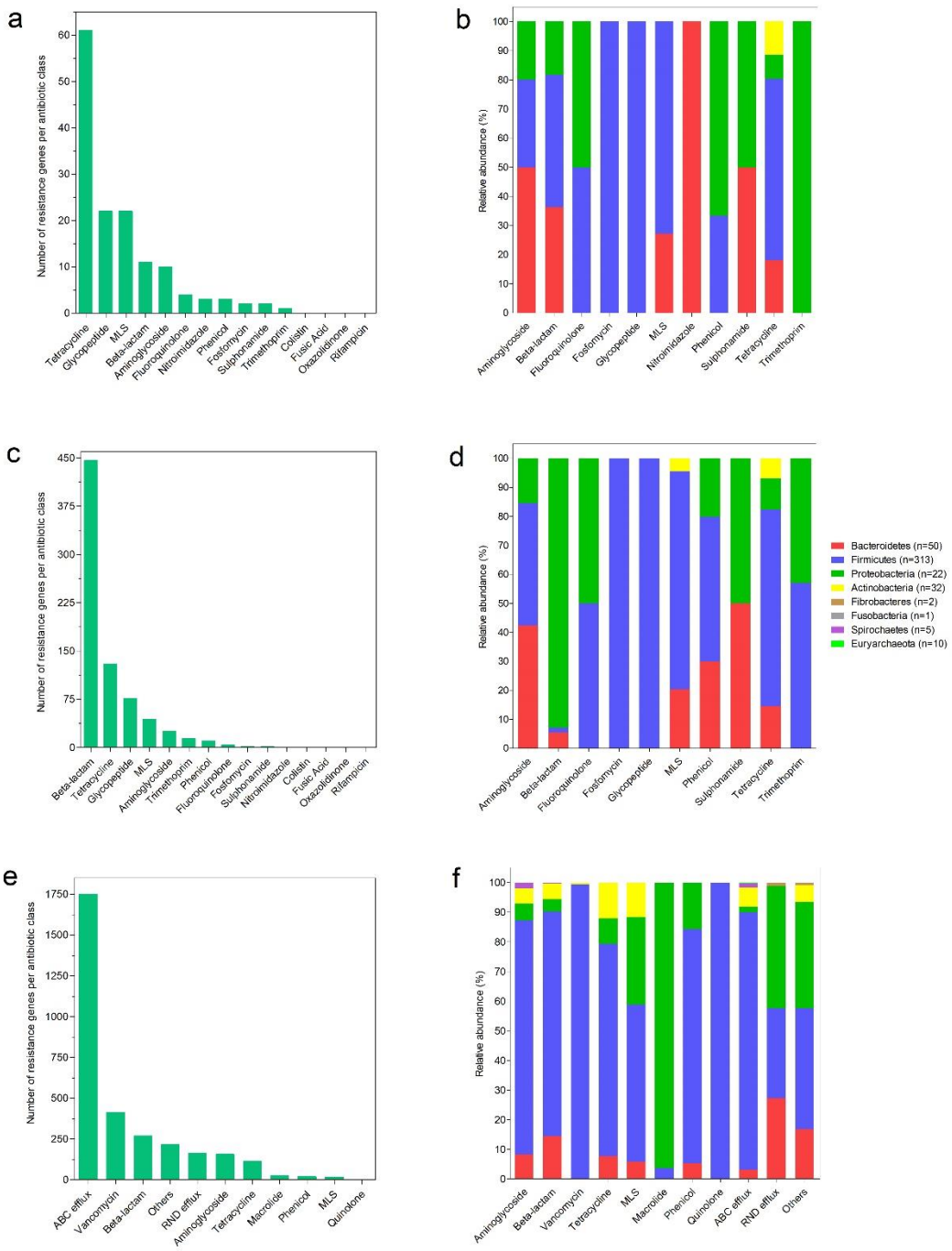


Figure 2. Number of resistance genes per antibiotic class and distribution of ARGs in the phyla analyzed in this study using ResFinder (a) and (b), ARG-ANNOT (c) and (d) and Resfams databases (e) and (f), respectively. The search was performed using BLASTn applying the following cut off parameters: 70% of sequence identity and 60% of sequence coverage.

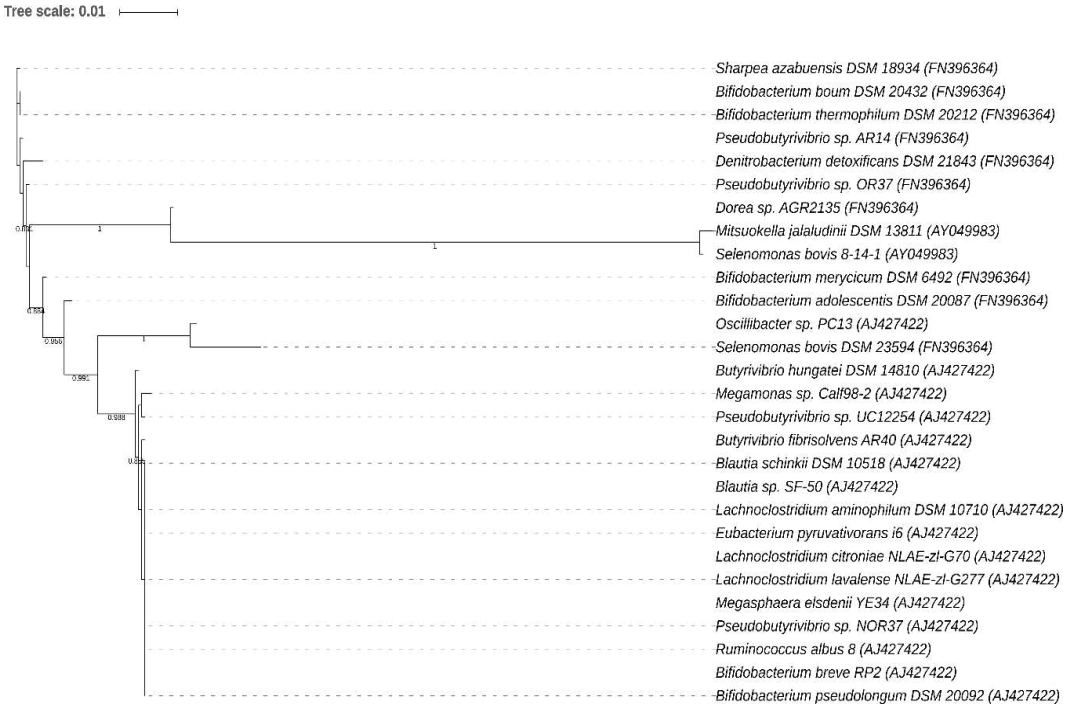


Figure 3. Phylogenetic tree of *tet*(W) sequences identified in the genomes of ruminal bacteria. The tree was generated using FastTree (Maximum Likelihood method, 1000 replications). Gene sequences were extracted from the ruminal microbial genomes using SAMtools. ResFinder database was used to identify the location of each *tet*(W) gene in the microbial genomes. Only bootstrap values greater than 0.7 are shown.

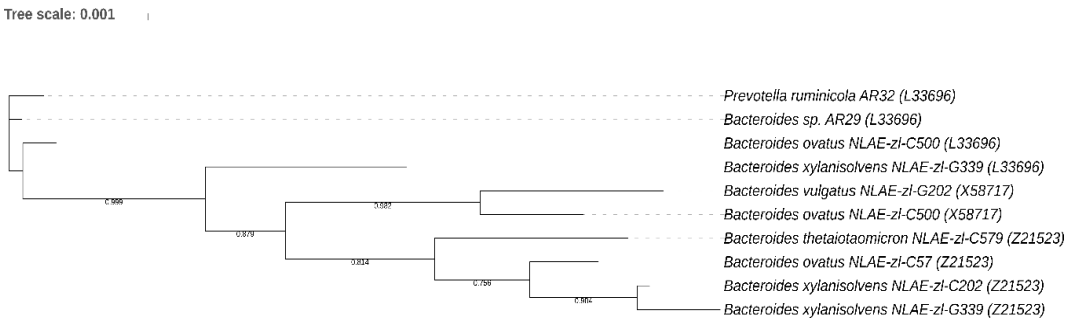


Figure 4. Phylogenetic tree of *tet*(Q) sequences identified in the genomes of ruminal bacteria. The tree was generated using FastTree (Maximum Likelihood method, 1000 replications). Gene sequences were extracted from the ruminal microbial genomes using SAMtools. ResFinder database was used to identify the location of each *tet*(Q) gene in the microbial genomes. Only bootstrap values greater than 0.7 are shown.

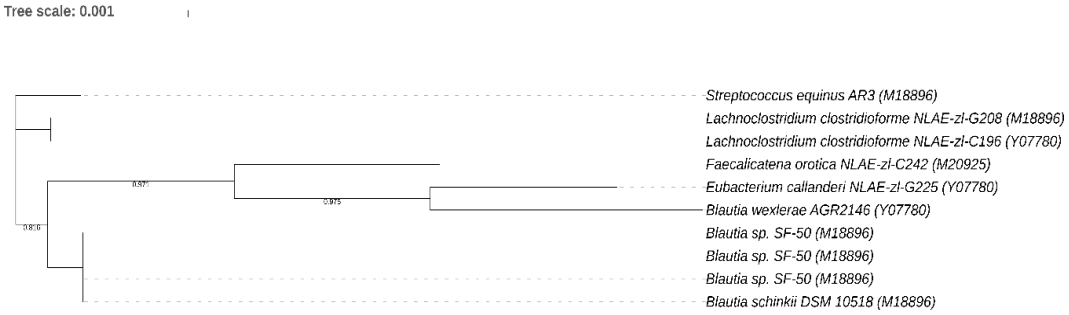


Figure 5. Phylogenetic tree of *tet*(O) sequences identified in the genomes of ruminal bacteria. The tree was generated using FastTree (Maximum Likelihood method, 1000 replications). Gene sequences were extracted from the ruminal microbial genomes using SAMtools. ResFinder database was used to identify the location of each *tet*(O) gene in the microbial genomes. Only bootstrap values greater than 0.7 are shown.

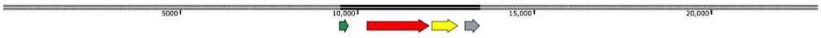
Bifidobacterium adolescentis DSM 20087

Bifidobacterium boum DSM 20432

Bifidobacterium breve RP2

Bifidobacterium merycicum DSM 6492

Bifidobacterium pseudolongum DSM 20092



Bifidobacterium thermophilum DSM 20212

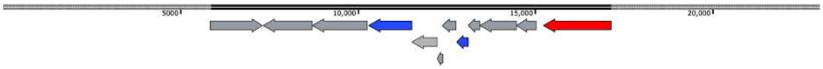
Blautia schinkii DSM 10518

Blautia sp. SF50

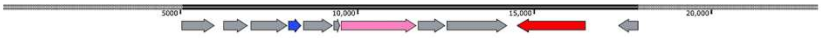
Butyrivibrio fibrisolvens AR40

Butyrivibrio hungatei DSM 14810

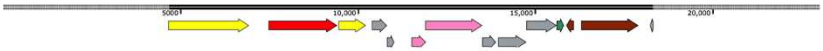
Denitrobacterium detoxificans DSM 21843



Dorea sp. AGR2135



Eubacterium pyruvativorans i6



Lachnodostridium aminophilum DSM 10710

(continue)

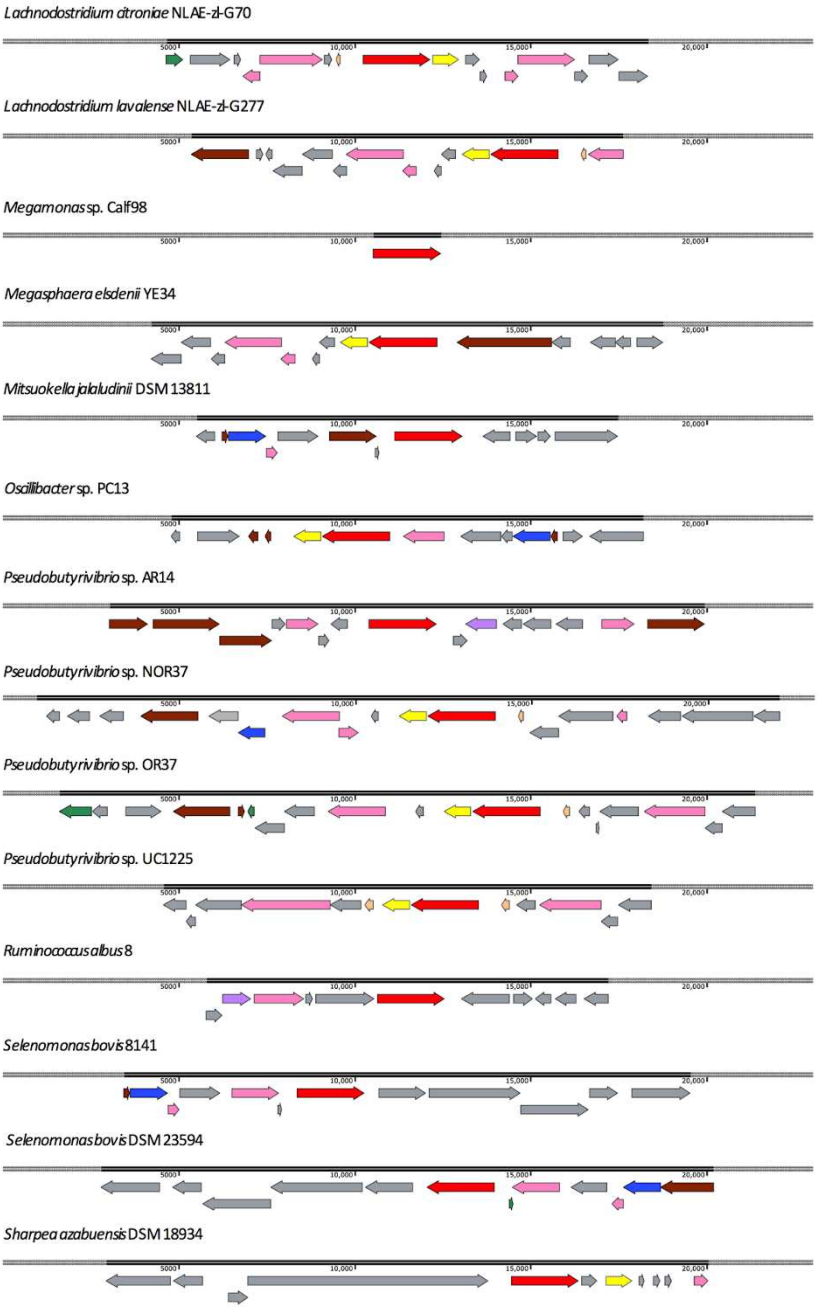


Figure 6. Genetic context of the *tet*(W) gene distributed in ruminal microbial genomes. The genes are represented by arrows classified and colored according to their function. Blue: phage proteins, yellow: methyltransferases; green: transposon/integron proteins; pink: conjugations proteins; red: *tet*(W); purple: other ARGs; brown: recombinase/endonuclease/excisionase; beige: Maff-2 protein, gray:

genes encoding other proteins. The graphic representation of these flanking genes was performed using the software SnapGene 2.3.2.

Figure 7. Venn diagram showing the number of ARGs (ARG-ANNOT, ResFinder and Resfams) or resistance phenotypes (detected *in vitro*) identified in the 26 ruminal microbial genomes or corresponding cultures. The Venn diagram was constructed using the tools available in the "Bioinformatics & Evolutionary Genomics" website (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Table 1. Antibiotic Resistant Genes (ARGs) identified using ResFinder that were shared by different species of ruminal bacteria.

Genome	Gene	Resistance	Identity (%)	Lenght (pb)	Acession number
Bacteroides thetaiotaomicron NLAE-zl-C579	*aadE*	aminoglycoside	100	867/867	KF864551
Blautia schinkii DSM 10518	*aadE*	aminoglycoside	100	867/867	KF864551
Oscillibacter sp. PC13	*aadE*	aminoglycoside	100	867/867	KF864551
Pseudobutyrivibrio sp. AR14	*aadE*	aminoglycoside	100	867/867	KF864551
Bacteroides thetaiotaomicron NLAE-zl-C579	*aph*(3')-III	aminoglycoside	100	795/795	M26832
Bacteroides xylanisolvens NLAE-zl-C202	*aph*(3')-III	aminoglycoside	100	795/795	M26832
Bacteroides thetaiotaomicron NLAE-zl-C579	*ant*(6)-Ia	aminoglycoside	100	909/909	AF330669
Bacteroides xylanisolvens NLAE-zl-C202	*ant*(6)-Ia	aminoglycoside	100	909/909	AF330669
Bacteroides ovatus NLAE-zl-C500	*cepA*	aminoglycoside	80.7	771/903	FR688022
Bacteroides xylanisolvens NLAE-zl-C202	*cepA*	aminoglycoside	79.8	773/903	FR688022
Selenomonas bovis DSM 23594	*blaACI*-1	beta-lactam	99.7	855/855	AJ007350
Selenomonas ruminantium WCT3	*blaACI*-1	beta-lactam	98.9	554/855	AJ007350
Succiniclasticum ruminis DSM11005	*blaACI*-1	beta-lactam	100	855/855	AJ007350
Enterococcus casseliflavus NLAE-zl-C414	*vanT*-C	vancomycin	92.9	2103/2103	EU151752
Enterococcus casseliflavus NLAE-zl-G268	*vanT*-C	vancomycin	92.4	2103/2103	EU151752
Enterococcus casseliflavus NLAE-zl-C414	*vanR*-C	vancomycin	92.9	696/696	EU151753
Enterococcus casseliflavus NLAE-zl-G268	*vanR*-C	vancomycin	92.7	696/696	EU151753
Enterococcus casseliflavus NLAE-zl-C414	*vanS*-C	vancomycin	90.7	1016/1065	EU151753
Enterococcus casseliflavus NLAE-zl-G268	*vanS*-C	vancomycin	90.9	1016/1065	EU151753
Enterococcus casseliflavus NLAE-zl-C414	*vanXY*-C	vancomycin	98.4	573/573	EU151754
Enterococcus casseliflavus NLAE-zl-G268	*vanXY*-C	vancomycin	99.5	573/573	EU151754
Enterococcus casseliflavus NLAE-zl-G268	*vanC*	vancomycin	98.6	1053/1053	EU151754
Enterococcus casseliflavus NLAE-zl-C414	*vanC*	vancomycin	99	1053/1053	EU151754
Proteus mirabilis NLAE-zl-C285	*cat*	phenicol	98.5	655/654	M11587

Proteus mirabilis NLAE-zl-G534	*cat*	phenicol	98	655/654	M11587
Staphylococcus epidermidis AG42	*norA*	fluorquinolone	77.6	854/1167	M97169
Staphylococcus epidermidis NLAE-zl-G239	*norA*	fluorquinolone	77.5	854/1167	M97169
Acetitomaculum ruminis 5522	*lnu*(C)	lincosamine	99.2	495/495	AY928180
Acetitomaculum ruminis 5522	*lnu*(C)	lincosamine	99.2	495/495	AY928180
Acetitomaculum ruminis 5522	*lnu*(C)	lincosamine	99.2	495/495	AY928180
Megamonas sp. Calf98	*lnu*(C)	lincosamine	99.2	495/495	AY928180
Porphyromonadaceae bacterium KHP3R9	*lnu*(D)	lincosamine	80.4	428/495	EF452177
Proteiniclasticum ruminis DSM24773	*lnu*(D)	lincosamine	83.4	495/495	EF452177
Prevotella ruminicola D31d	*nimJ*	metronidazole	77.8	311/498	NZ_JH815495
Prevotella sp. KHP1	*nimJ*	metronidazole	82.3	340/498	NZ_JH815495
Prevotella sp. RM4	*nimJ*	metronidazole	82.3	340/498	NZ_JH815495
Bacteroides ovatus NLAE-zl-C500	*mef*(A)	MLS	94.9	1218/1218	AF227521
Bacteroides xylanisolvens NLAE-zl-C202	*mef*(A)	MLS	95.2	1218/1218	AF227521
Bacteroides xylanisolvens NLAE-zl-G339	*mef*(A)	MLS	94.9	1218/1218	AF227521
Clostridium lundense DSM 17049	*mef*(A)	MLS	95.7	1218/1218	AF227521
Eubacterium callanderi NLAE-zl-G225	*mef*(A)	MLS	95.3	1218/1218	AF227521
Bacteroides ovatus NLAE-zl-C500	*sul2*	MLS	100	816/816	GQ421466
Escherichia coli PA-3	*sul2*	MLS	100	816/816	GQ421466
Blautia schinkii DSM 10518	*tet*(O)	tetracycline	99.8	1920/1920	M18896
Blautia sp. SF50	*tet*(O)	tetracycline	99.8	1920/1920	M18896
Blautia wexlerae AGR2146	*tet*(O)	tetracycline	99.3	1920/1916	Y07780
Eubacterium callanderi NLAE-zl-G225	*tet*(O)	tetracycline	100	1920/1920	Y07780
Lachnoclostridium clostridioforme NLAE-zl-C196	*tet*(O)	tetracycline	99.7	1920/1920	M18896
Lachnoclostridium clostridioforme NLAE-zl-G208	*tet*(O)	tetracycline	99.7	1920/1920	M18896
Streptococcus equinus AR3	*tet*(O)	tetracycline	99.6	1920/1920	M18896
Bifidobacterium breve RP2	*tet*(W)	tetracycline	100	1920/1920	AJ427422

Bifidobacterium adolescentis DSM 20087	*tet*(W)	tetracycline	98.9	1920/1920	FN396364
Bifidobacterium boum DSM 20432	*tet*(W)	tetracycline	99.1	1920/1920	FN396364
Bifidobacterium merycicum DSM 6492	*tet*(W)	tetracycline	98.7	1920/1920	FN396364
Bifidobacterium pseudologum DSM 20092	*tet*(W)	tetracycline	100	1920/1920	AJ427422
Bifidobacterium thermophilum DSM 20212	*tet*(W)	tetracycline	99.1	1920/1920	FN396364
Blautia schinkii DSM 10518	*tet*(W)	tetracycline	100	1920/1920	AJ427422
Blautia sp. SF50	*tet*(W)	tetracycline	100	1920/1920	AJ427422
Butyrivibrio fibrisolvens AR40	*tet*(W)	tetracycline	99.9	1920/1920	AJ427422
Butyrivibrio hungatei DSM 14810	*tet*(W)	tetracycline	99.9	1920/1920	AJ427422
Lachnoclostridium aminophilum DSM 10710	*tet*(W)	tetracycline	100	1920/1920	AJ427422
Denitrobacterium detoxificans DSM 21843	*tet*(W)	tetracycline	98.8	1920/1920	FN396364
Dorea sp. AGR2135	*tet*(W)	tetracycline	97.6	1920/1904	FN396364
Eubacterium pyruvativorans i6	*tet*(W)	tetracycline	100	1920/1920	AJ427422
Lachnoclostridium citroniae NLAE-zl-G70	*tet*(W)	tetracycline	100	1920/1920	AJ427422
Lachnoclostridium lavalense NLAE-zl-G277	*tet*(W)	tetracycline	100	1920/1920	AJ427422
Megamonas sp. Calf98	*tet*(W)	tetracycline	100	1912/1920	AJ427422
Megasphaera elsdenii YE34	*tet*(W)	tetracycline	100	1920/1920	AJ427422
Mitsuokella jalaludinii DSM 13811	*tet*(W)	tetracycline	94.9	1904/1920	AY049983
Oscillibacter sp. PC13	*tet*(W)	tetracycline	97.9	1920/1920	AJ427422
Pseudobutyrivibrio sp. AR14	*tet*(W)	tetracycline	99.1	1920/1920	FN396364
Pseudobutyrivibrio sp. NOR37	*tet*(W)	tetracycline	100	1920/1920	AJ427422
Pseudobutyrivibrio sp. OR37	*tet*(W)	tetracycline	99.1	1920/1920	FN396364
Pseudobutyrivibrio sp. UC1225	*tet*(W)	tetracycline	99.9	1920/1920	AJ427422
Ruminococcus albus 8	*tet*(W)	tetracycline	100	1920/1920	AJ427422
Selenomonas bovis DSM 23594	*tet*(W)	tetracycline	97.5	1904/1920	FN396364
Selenomonas bovis 8141	*tet*(W)	tetracycline	94.9	1904/1920	AY049983
Sharpea azabuensis DSM 18934	*tet*(W)	tetracycline	99.1	1920/1920	FN396364

Bacteroides ovatus NLAE-zl-C500	*tet*(Q)	tetracycline	99.6	1926/1926	L33696
Bacteroides ovatus NLAE-zl-C500	*tet*(Q)	tetracycline	98.8	1926/1926	X58717
Bacteroides ovatus NLAE-zl-C57	*tet*(Q)	tetracycline	99.2	1974/1974	Z21523
Bacteroides sp. AR29	*tet*(Q)	tetracycline	99.8	1926/1926	L33696
Bacteroides thetaiotaomicron NLAE-zl-C579	*tet*(Q)	tetracycline	98.3	1323/1974	Z21523
Bacteroides vulgatus NLAE-zl-G202	*tet*(Q)	tetracycline	99.9	1926/1926	X58717
Bacteroides xylanisolvens NLAE-zl-C202	*tet*(Q)	tetracycline	100	1974/1974	Z21523
Bacteroides xylanisolvens NLAE-zl-G339	*tet*(Q)	tetracycline	98.2	1926/1926	L33693
Bacteroides xylanisolvens NLAE-zl-G339	*tet*(Q)	tetracycline	99.6	1974/1974	Z21523
Prevotella ruminicola AR32	*tet*(Q)	tetracycline	100	1926/1926	L33696
Proteus mirabilis NLAE-zl-C285	*tet*(J)	tetracycline	99.2	1197/1197	ACLE01000065
Proteus mirabilis NLAE-zl-G534	*tet*(J)	tetracycline	99.3	1197/1197	ACLE01000065

Table 2. Genomes of ruminal bacteria and archaea used in this study (data collected from March 2017 to August 2017).

Strain	Genome Size (Mb)	Source	Accession Number	Database
Acetitomaculum ruminis DSM 5522	3.1	Cow rumen/USA	SAMN05216249	BioSample/ Hungate1000*
Acidaminococcus fermentans pGA-4	2.4	Cow rumen/NZ	FOWJ00000000.1	Genbank
Acidaminococcus fermentans WCC6	2.3	Cow rumen/NZ	SAMN05216495	BioSample/ Hungate1000*
Acinetobacter sp. DSM 11652	3.1	Sheep rumen/Germany	SAMN05216500	BioSample/ Hungate1000*
Actinobacillus succinogenes 130Z	2.3	Cow rumen/USA	NC_009655. 1	Genbank
Actinomyces denticolens PA	2.8	Cow rumen/NZ	SAMN05216246	BioSample/ Hungate1000*
Actinomyces nasicola KPR-1	2.5	Cow rumen/NZ	FNQV00000000.1	Genbank
Actinomyces ruminicola DSM 27982	3.1	Cow rumen/China	NZ_FNIM00000000. 1	Genbank
Actinomyces ruminicola KPR-7B	3.3	Cow rumen/NZ	FNHU00000000.1	Genbank
Allisonella histaminiformans DSM 15230	1.7	Cow rumen/USA	PRJNA254896	BioProject/ Hungate1000*
Alysiella crassa DSM 2578	2.7	Sheep saliva/Australia	SAMN02746063	BioSample/ Hungate1000*
Anaerovibrio lipolyticus LB2005	2.7	Cow rumen/NZ	JHYA00000000.1	Genbank
Anaerovibrio sp. RM50	2.8	Cow rumen/Japan	JHWV00000000.1	Genbank
Bacillus cereus KPR-7A	5.8	Cow rumen/NZ	SAMN04487767	BioSample/ Hungate1000*
Bacillus licheniformis VTM3R78	4.1	Moose rumen/USA	NZ_FOFE00000000 .1	Genbank
Bacillus sp. MB2021	5	Cow rumen/NZ	JNJJ00000000.1	Genbank

Bacteroidales bacterium Ga6A1	3.8	Cow rumen/NZ	JNLA01000001.1	Genbank
Bacteroidales bacterium Ga6A2	3.9	Cow rumen/NZ	JNKX00000000.1	Genbank
Bacteroidales bacterium WCE2008	2.4	Cow rumen/NZ	NZ_FUZM00000000.1	Genbank
Bacteroides ovatus NLAE-zl-C57	6	Cow faeces enrichment/USA	SAMN05192582	BioSample/ Hungate1000*
Bacteroides ovatus NLAE-zl-C500	5.9	Cow faeces enrichment/USA	NZ_FMYE00000000.1	Genbank
Bacteroides sp. AR20	6.1	Sheep rumen/Australia	FOBY00000000.1	Genbank
Bacteroides sp. AR29	4.4	Sheep rumen/Australia	SAMN02910431	BioSample/ Hungate1000*
Bacteroides thetaiotaomicron KPPR-3	6.2	Cow rumen/NZ	SAMN02910322	BioSample/ Hungate1000*
Bacteroides thetaiotaomicron NLAE-zl-C579	6.3	Cow faeces enrichment/USA	FNVL00000000.1	Genbank
Bacteroides vulgatus NLAE-zl-G202	5	Goat faeces enrichment/USA	NZ_FOBA00000000.1	Genbank
Bacteroides xylanisolvens NLAE-zl-C202	5.9	Cow faeces enrichment/USA	FOUM00000000.1	Genbank
Bacteroides xylanisolvens NLAE-zl-G339	6.1	Goat faeces enrichment/USA	NZ_FNRP00000000.1	Genbank
Basfia succiniciproducens DSM 22022	2.2	Cow rumen/Switzerland	FMUQ00000000.1	Genbank
Basfia succiniciproducens KPR-2	2.3	Cow rumen/NZ	FOFH00000000.1	Genbank
Basfia succiniproducens MBEL55E	2.3	Cow rumen/Korea	NC_006300.1	Genbank
Bifibacterium choerinum AGR2158	2.2	Calf faeces/NZ	SAMN02441493	BioSample/ Hungate1000*
Bifidobacterium adolescentis DSM 20087	2.1	Cow rumen/Italy	JNKM00000000.1	Genbank
Bifidobacterium bifidum Calf96	2.2	Calf faeces/NZ	SAMN05216254	BioSample/ Hungate1000*

Bifidobacterium boum DSM 20432	2.2	Cow rumen/Italy	JHWO00000000.1	Genbank
Bifidobacterium breve RP2	2.3	Calf faeces/NZ	SAMN05216468	BioSample/ Hungate1000*
Bifidobacterium longum AGR2137	2.3	Calf faeces/NZ	ATWX00000000.1	Genbank
Bifidobacterium merycicum DSM 6492	2.3	Cow rumen/Italy	JDTL00000000.1	Genbank
Bifidobacterium pseudolongum AGR2145	2	Calf faeces/NZ	NZ_ATWW00000000.1	Genbank
Bifidobacterium pseudolongum DSM 20092	1.9	Cow rumen/Italy	JHWN00000000.1	Genbank
Bifidobacterium ruminantium DSM 6489	2.2	Cow rumen/Italy	JHWQ00000000.1	Genbank
Bifidobacterium thermophilum DSM 20212	2.3	Cow rumen/Italy	JHWM00000000.1	Genbank
Blautia schinkii DSM 10518	6.7	Lamb rumen/France	JNKJ00000000.1	Genbank
Blautia sp. SF-50	3.7	Cow rumen/USA	SAMN02910433	BioSample/ Hungate1000*
Blautia wexlerae AGR2146	3.6	Calf faeces/NZ	AUJF00000000.1	Genbank
Butyrivibrio fibrisolvens AB2020	4.7	Cow rumen/NZ	ATVZ00000000.1	Genbank
Butyrivibrio fibrisolvens AR40	5	Sheep rumen/Australia	NZ_FOGJ00000000.1	Genbank
Butyrivibrio fibrisolvens DSM 3071	4.8	Cow rumen/USA	NZ_FQXK00000000.1	Genbank
Butyrivibrio fibrisolvens FE2007	4.3	Cow rumen/NZ	AUJW00000000.1	Genbank
Butyrivibrio fibrisolvens MD2001	4.7	Cow rumen/NZ	AUKD00000000.1	Genbank
Butyrivibrio fibrisolvens ND3005	4.5	Cow rumen/NZ	NZ_ATVY00000000.1	Genbank
Butyrivibrio fibrisolvens TB	4.4	Cow rumen/NZ	SAMN02910382	BioSample/ Hungate1000*
Butyrivibrio fibrisolvens WTE3004	4.7	Cow rumen/NZ	AUJV00000000.1	Genbank
Butyrivibrio fibrisolvens YRB2005	4.7	Cow rumen/NZ	AUJA00000000.1	Genbank
Butyrivibrio hungatei DSM 14810	3.4	Sheep rumen/Czech republic	NZ_FRDH00000000.1	Genbank

Butyrivibrio hungatei NK4A153	3.4	Sheep rumen/NZ	AUJY00000000.1	Genbank
Butyrivibrio hungatei XBD2006	3.4	Cow rumen/NZ	PRJNA254909	BioProject/ Hungate1000*
Butyrivibrio proteoclasticus B316	3.6	Cow rumen/NZ	NC_014387.1	Genbank
Butyrivibrio proteoclasticus FD2007	3.9	Cow rumen/NZ	JHYH00000000.1	Genbank
Butyrivibrio proteoclasticus P18	4.2	Sheep rumen/UK	NZ_FOXO00000000 .1	Genbank
Butyrivibrio proteoclasticus P6B7	3.9	Cow rumen/NZ	PRJNA223495	BioProject/ Hungate1000*
Butyrivibrio sp. AC2005	5.1	Cow rumen/NZ	GCA_000424145.1	Genbank
Butyrivibrio sp. AD3002	4.3	Cow rumen/NZ	ATVV00000000.1	Genbank
Butyrivibrio sp. AE2005	3.7	Cow rumen/NZ	JMMD00000000.1	BioSample/ Hungate1000*
Butyrivibrio sp. AE2015	3.7	Cow rumen/NZ	ATVR00000000.1	Genbank
Butyrivibrio sp. AE2032	3.7	Cow rumen/NZ	JNLF00000000.1	Genbank
Butyrivibrio sp. AE3003	3.5	Cow rumen/NZ	JMLX00000000.1	Genbank
Butyrivibrio sp. AE3004	4.5	Cow rumen/NZ	JNLQ00000000.1	Genbank
Butyrivibrio sp. AE3006	4.1	Cow rumen/NZ	AUJJ00000000.1	Genbank
Butyrivibrio sp. AE3009	4.2	Cow rumen/NZ	ATVS00000000.1	Genbank
Butyrivibrio sp. FC2001	4.6	Cow rumen/NZ	NZ_AUJH00000000 .1	Genbank
Butyrivibrio sp. FCS006	3.8	Cow rumen/NZ	AUKB00000000.1	Genbank
Butyrivibrio sp. FCS014	4.1	Cow rumen/NZ	GCA_000526935.1	Genbank
Butyrivibrio sp. INIIa14	4.2	Llama forestomach/Argentina	PRJNA254902	BioProject/ Hungate1000*
Butyrivibrio sp. INIIa16	4.8	Llama forestomach/Argentina	SAMN02910263	BioSample/ Hungate1000*
Butyrivibrio sp. INIIa18	3.3	Llama forestomach/Argentina	PRJNA243945	BioProject/ Hungate1000*

Butyrivibrio sp. INIla21	3.4	Llama forestomach/Argentina	PRJNA254905	BioProject/ Hungate1000*
Butyrivibrio sp. LB2008	3.7	Cow rumen/NZ	JHXZ00000000.1	Genbank
Butyrivibrio sp. LC3010	4.6	Cow rumen/NZ	AUJU00000000.1	Genbank
Butyrivibrio sp. M55	3.7	Camel forestomach/Australia	GCA_900116865.1	Genbank
Butyrivibrio sp. MB2005	4.2	Cow rumen/NZ	AUJP00000000.1	Genbank
Butyrivibrio sp. MC2013	3.8	Cow rumen/NZ	AUKE00000000.1	Genbank
Butyrivibrio sp. MC2021	4.4	Cow rumen/NZ	SAMN02744002	BioSample/ Hungate1000*
Butyrivibrio sp. NC2002	3.4	Cow rumen/NZ	JNKZ00000000.1	Genbank
Butyrivibrio sp. NC2007	4	Cow rumen/NZ	ATWY00000000.1	Genbank
Butyrivibrio sp. NC3005	3.9	Cow rumen/NZ	AUKC00000000.1	Genbank
Butyrivibrio sp. OB235	4.7	Cow rumen/Canada	FOBE00000000.1	Genbank
Butyrivibrio sp. Su6	3.7	Cow rumen/NZ	SAMN02910276	BioSample/ Hungate1000*
Butyrivibrio sp. VCB2001	4	Cow rumen/NZ	AUIZ00000000.1	Genbank
Butyrivibrio sp. VCB2006	4	Cow rumen/NZ	ATVX00000000.1	Genbank
Butyrivibrio sp. VCD2006	4.7	Cow rumen/NZ	AUIX00000000.1	Genbank
Butyrivibrio sp. WCD2001	4.3	Cow rumen/NZ	AUJZ00000000.1	Genbank
Butyrivibrio sp. WCD3002	4.3	Cow rumen/NZ	AUJO00000000.1	Genbank
Butyrivibrio sp. WCE2006	4.5	Cow rumen/NZ	JNKH00000000.1	Genbank
Butyrivibrio sp. XBB1001	3.9	Cow rumen/NZ	AUKA00000000.1	Genbank
Butyrivibrio sp. XPD2002	4.4	Cow rumen/NZ	AUIY00000000.1	Genbank
Butyrivibrio sp. XPD2006	3.9	Cow rumen/NZ	ATVT00000000.1	Genbank
Butyrivibrio sp. YAB3001	4.6	Cow rumen/NZ	SAMN02910398	BioSample/ Hungate1000*
Cellulomonas sp. KH9	4.2	Cow rumen/NZ	FOSE00000000.1	Genbank

Citrobacter sp. NLAE-zl-C269	5	Cow faeces enrichment/USA	SAMN05216502	BioSample/ Hungate1000*
Clostridiales bacterium R-7	3.3	Sheep rumen/Japan	FZQO01000001.1	Genbank
Clostridiales bacterium WTE2008	3.4	Cow rumen/NZ	SAMN06297397	BioSample/ Hungate1000*
Clostridium algidicarnis B3	3.1	Cow faeces/NZ	JNLN00000000.1	Genbank
Clostridium beijerinkii HUN142	6.1	Cow rumen/NZ	JHXK00000000.1	Genbank
Clostridium butyricum AGR2140	4.6	Calf faeces/NZ	AUJN00000000.1	Genbank
Clostridium cadaveris AGR2141	3.5	Calf faeces/NZ	AUJL00000000.1	Genbank
Clostridium cadaveris NLAE-zl-G419	3.5	Goat faeces enrichment/USA	NZ_FOOE00000000 .1	Genbank
Clostridium cochlearium NLAE-zl-C224	2.4	Cow faeces enrichment/USA	SAMN05216497	BioSample/ Hungate1000*
Clostridium intestinale DSM 6191	4.6	Cow faeces/Japan	SAMN02745941	BioSample/ Hungate1000*
Clostridium lundense DSM 17049	4.8	Cow rumen/Sweden	JHVC00000000.1	Genbank
Clostridium paraputrificum AGR2156	3.6	Calf faeces/NZ	AUJC00000000.1	Genbank
Corynebacterium vitaeruminis DSM 20294	2.9	Cow rumen/USA	NZ_CP004353.1	Genbank
Corynebacterium vitaeruminis GA6A13	2.9	Cow rumen/NZ	JNKV00000000.1	Genbank
Cutibacterium acnes NLAE-zl-G260	2.5	Goat faeces enrichment/USA	NZ_FNGJ00000000 .1	Genbank
Denitrobacterium detoxificans DSM 21843	2.4	Cow rumen/USA	NZ_FOEC00000000 .1	Genbank
Desulfotomaculum ruminis DSM 2154	4	Sheep rumen/UK	CP002780.1	Genbank
Desulfovibrio desulfuricans ATCC 27774	2.9	Sheep rumen/USA	SAMN00001882	BioSample/ Hungate1000*
Desulfovibrio desulfuricans DSM 7057	3.2	Cow rumen/Germany	NZ_FPIW00000000. 1	Genbank
Desulfovibrio legallii KHC7	2.7	Cow rumen/NZ	SAMN05192586	BioSample/

				Hungate1000*
Dorea sp. AGR2135	3.5	Calf faeces/NZ	ATVU00000000	Genbank
Enterobacter sp. KPR-6	4.5	Cow rumen/NZ	FOYH00000000.1	Genbank
Enterococcus casseliflavus NLAE-zl-C414	3.6	Cow faeces enrichment/USA	SAMN05216513	BioSample/ Hungate1000*
Enterococcus casseliflavus NLAE-zl-G268	3.7	Goat faeces enrichment/USA	NZ_FOMP00000000.1	Genbank
Enterococcus faecalis 68A	3	Sheep rumen/NZ	JOOC00000000.1	Genbank
Enterococcus faecalis VTM1R91	3.1	Moose rumen/USA	NZ_FNIP01000007.1	Genbank
Enterococcus gallinarum SKF1	3.5	Sheep rumen/NZ	JNLR00000000.1	Genbank
Enterococcus mundtii C2	3.3	Cow rumen/NZ	NZ_FOUC01000012.1	Genbank
Enterococcus sp. KPPR-6	4.5	Cow rumen/NZ	FOHR00000000.1	Genbank
Erysipelatoclostridium innocuum NLAE-zl-C381	4.2	Cow faeces enrichment/USA	SAMN05216507	BioSample/ Hungate1000*
Erysipelatoclostridium innocuum NLAE-zl-G197	4.6	Goat faeces enrichment/USA	SAMN04487929	BioSample/ Hungate1000*
Erysipelotrichaceae bacterium NK3D112	3.1	Sheep rumen/NZ	JNJQ00000000.1	Genbank
Escherichia coli PA-3	4.9	Cow rumen/NZ	SAMN04487822	BioSample/ Hungate1000*
Eubacterium callanderi NLAE-zl-G225	4.4	Goat faeces enrichment/USA	NZ_FOWI00000000.1	Genbank
Eubacterium celullosolvens LD2006	3.3	Cow rumen/NZ	JHXY00000000.1	Genbank
Eubacterium oxidoreducens DSM 3217	2.9	Cow rumen/USA	FMXR00000000.1	Genbank
Eubacterium pyruvativorans i6	2.2	Sheep rumen/UK	NZ_FNBF00000000.1	Genbank
Eubacterium pyruvativorans KHGC13	2.3	Cow rumen/NZ	SAMN05216508	BioSample/ Hungate1000*

Eubacterium pyruvativorans KHPC4	2.3	Cow rumen/NZ	SAMN05216515	BioSample/ Hungate1000*
Eubacterium ruminantium 2388	2.9	Sheep rumen/UK	NZ_FNHR00000000.1	Genbank
Eubacterium ruminantium FB3002	3.5	Cow rumen/NZ	FNUU01000001.1	Genbank
Eubacterium ruminatium HUN269	2.9	Cow rumen/NZ	SAMN05660484	BioSample/ Hungate1000*
Eubacterium sp. AB3007	2.3	Cow rumen/NZ	NZ_JIAD00000000.1	Genbank
Faecalicatena contorta NLAE-zl-C134	4.7	Cow faeces enrichment/USA	SAMN05216529	BioSample/ Hungate1000*
Faecalicatena orotica NLAE-zl-C242	5.7	Cow faeces enrichment/USA	SAMN05216536	BioSample/ Hungate1000*
Fibrobacter succinogenes HM2	3.5	Sheep rumen/USA	SRP092671	BioSample/ Hungate1000*
Fibrobacter succinogenes S85	3.8	Cow rumen/USA	CP002158.1	Genbank
Fusobacterium necrophorum HUN048	2	Cow rumen/NZ	JHWT00000000.1	Genbank
Kandleria vitulina DSM 20405	2.1	Calf rumen/UK	JNKN00000000.1	Genbank
Kandleria vitulina KH4T7	2.1	Cow rumen/NZ	SAMN05216514	BioSample/ Hungate1000*
Kandleria vitulina MC3001	2	Cow rumen/NZ	JHWI00000000.1	Genbank
Kandleria vitulina S3b	2.1	Sheep rumen/NZ	NZ_FNNF00000000.1	Genbank
Kandleria vitulina WCC7	2.1	Cow rumen/NZ	SAMN05216520	BioSample/ Hungate1000*
Kandleria vitulina WCE2011	2	Cow rumen/NZ	JHXA00000000.1	Genbank
Lachnobacterium bovis AE2004	2.9	Cow rumen/NZ	NZ_JHWB00000000.1	Genbank
Lachnobacterium bovis C6A12	2.6	Cow rumen/NZ	JHWP00000000.1	Genbank

Lachnobacterium bovis DSM 14045	2.7	Cow rumen/Canada	SAMN02910414	BioSample/ Hungate1000*
Lachnobacterium bovis NK4B19	2.5	Sheep rumen/NZ	ATWB00000000.1	Genbank
Lachnobacterium bovis S1b	2.5	Sheep rumen/NZ	NZ_FOGW00000000.1	Genbank
Lachnoclostridium aerotolerans DSM 5434	4.7	Sheep rumen/South Africa	JHWJ00000000.1	Genbank
Lachnoclostridium aminophilum DSM 10710	3.1	Cow rumen/USA	SAMN02745353	BioSample/ Hungate1000*
Lachnoclostridium aminophilum KH1P1	3.2	Cow rumen/NZ	SAMN04487771	BioSample/ Hungate1000*
Lachnoclostridium citroniae NLAE-zl-G70	6	Goat faeces enrichment/USA	NZ_FOZH00000000.1	Genbank
Lachnoclostridium clostridioforme AGR2157	4.9	Calf faeces/NZ	SAMN02440671	BioSample/ Hungate1000*
Lachnoclostridium clostridioforme ATCC 25537	5.5	Calf rumen/USA	NZ_FOOJ00000000.1	Genbank
Lachnoclostridium clostridioforme NLAE-zl-C196	5.2	Cow faeces enrichment/USA	SAMN05216521	BioSample/ Hungate1000*
Lachnoclostridium clostridioforme NLAE-zl-G208	5.2	Goat faeces enrichment/USA	SAMN05216528	BioSample/ Hungate1000*
Lachnoclostridium lavalense NLAE-zl-G277	6.4	Goat faeces enrichment/USA	NZ_FOIM00000000.1	Genbank
Lachnoclostridium polysaccharolyticum DSM 1801	3.5	Sheep rumen/South Africa	SAMN04487772	BioSample/ Hungate1000*
Lachnospira multipara D15d	2.8	Cow rumen/USA	SAMN05216537	BioSample/ Hungate1000*
Lachnospira multipara LB2003	2.6	Cow rumen/NZ	JNKW00000000.1	Genbank
Lachnospira multipara MC2003	2.6	Cow rumen/NZ	JHWY00000000.1	Genbank
Lachnospira mutipara ATCC 19207	2.9	Cow rumen/USA	AUJG00000000.1	Genbank

Lachnospira pectinoschiza M83	2.7	Camel forestomach/Australia	SAMN05216544	BioSample/ Hungate1000*
Lachnospiraceae bacterium A10	2.5	Cow rumen/NZ	FNYN00000000.1	Genbank
Lachnospiraceae bacterium AB2028	3.5	Cow rumen/NZ	JHYJ00000000.1	Genbank
Lachnospiraceae bacterium AC2012	2.6	Cow rumen/NZ	JNIN00000000.1	Genbank
Lachnospiraceae bacterium AC2014	2.8	Cow rumen/NZ	JNJE00000000.1	Genbank
Lachnospiraceae bacterium AC2028	2.8	Cow rumen/NZ	NZ_JNJD00000000.1	Genbank
Lachnospiraceae bacterium AC2029	3.9	Cow rumen/NZ	NZ_JNLK00000000.1	Genbank
Lachnospiraceae bacterium AC2031	3.2	Cow rumen/NZ	JIAB00000000.1	Genbank
Lachnospiraceae bacterium AC3007	2.9	Cow rumen/NZ	JHYK00000000.1	Genbank
Lachnospiraceae bacterium AD3010	3.5	Cow rumen/NZ	JHWU0000000.1	Genbank
Lachnospiraceae bacterium C10	3	Cow rumen/NZ	PRJNA254845	BioProject/ Hungate1000*
Lachnospiraceae bacterium C6A11	2.9	Cow rumen/NZ	JNKY00000000.1	Genbank
Lachnospiraceae bacterium C7	2.9	Cow rumen/NZ	FOPE00000000.1	Genbank
Lachnospiraceae bacterium FD2005	2.4	Cow rumen/NZ	JNKT00000000.1	Genbank
Lachnospiraceae bacterium FE2018	3.2	Cow rumen/NZ	JNKK00000000.1	Genbank
Lachnospiraceae bacterium G11	3.1	Cow rumen/Japan	FMWZ00000000.1	Genbank
Lachnospiraceae bacterium G41	2.6	Cow rumen/Japan	FNEU00000000.1	Genbank
Lachnospiraceae bacterium KH1P17	3.8	Cow rumen/NZ	SAMN06296386	BioSample/ Hungate1000*
Lachnospiraceae bacterium KH1T2	3.9	Cow rumen/NZ	SAMN05216390	BioSample/ Hungate1000*
Lachnospiraceae bacterium KHCPX20	3.2	Cow rumen/NZ	SAMN05216391	BioSample/ Hungate1000*
Lachnospiraceae bacterium LC2019	3.5	Cow rumen/NZ	NZ_FZRB00000000.1	Genbank

Lachnospiraceae bacterium MA2020	3.7	Cow rumen/NZ	JQKK00000000.1	Genbank
Lachnospiraceae bacterium MC2017	4.3	Cow rumen/NZ	JNJK00000000.1	Genbank
Lachnospiraceae bacterium MD2004	2.5	Cow rumen/NZ	JHWK00000000.1	Genbank
Lachnospiraceae bacterium NC2004	3.4	Cow rumen/NZ	NZ_JHXB00000000.1	Genbank
Lachnospiraceae bacterium NC2008	2.7	Cow rumen/NZ	JHWS00000000.1	Genbank
Lachnospiraceae bacterium ND2006	3.9	Cow rumen/NZ	SAMN02910369	BioSample/ Hungate1000*
Lachnospiraceae bacterium NE2001	3.7	Cow rumen/NZ	SAMN02910369	BioSample/ Hungate1000*
Lachnospiraceae bacterium NK3A20	3	Sheep rumen/NZ	SAMN02745687	BioSample/ Hungate1000*
Lachnospiraceae bacterium NK4A136	2.7	Sheep rumen/NZ	ATVW00000000.1	Genbank
Lachnospiraceae bacterium NK4A179	3.2	Sheep rumen/NZ	ATWC00000000	Genbank
Lachnospiraceae bacterium NK4A144	4.1	Sheep rumen/NZ	AUJT00000000.1	Genbank
Lachnospiraceae bacterium NLAE-zl-G231	7.3	Goat faeces enrichment/USA	SAMN05216405	BioSample/ Hungate1000*
Lachnospiraceae bacterium P6A3	3	Cow rumen/NZ	SAMN02841223	BioSample/ Hungate1000*
Lachnospiraceae bacterium P6B14	2.7	Cow rumen/NZ	JHWZ00000000.1	Genbank
Lachnospiraceae bacterium RM5	2.4	Cow rumen/Japan	AB730673.1	Genbank
Lachnospiraceae bacterium V9D3004	0.3	Cow rumen/NZ	SAMN02744022	BioSample/ Hungate1000*
Lachnospiraceae bacterium XBB1006	2.7	Cow rumen/NZ	SAMN02910358	BioSample/ Hungate1000*
Lachnospiraceae bacterium XBB2008	3.5	Cow rumen/NZ	SAMN02910292	BioSample/ Hungate1000*
Lachnospiraceae bacterium XBD2001	2.5	Cow rumen/NZ	SAMN02910301	BioSample/ Hungate1000*

Lachnospiraceae bacterium XPB1003	3.2	Cow rumen/NZ	FMVK00000000.1	Genbank
Lachnospiraceae bacterium YSB2008	2.7	Cow rumen/NZ	JHWR00000000.1	Genbank
Lachnospiraceae bacterium YSD2013	3.2	Cow rumen/NZ	SAMN02910339	BioSample/ Hungate1000*
Lactobacillus brevis AG48	2.6	Sheep rumen/NZ	GCF_000526755.1	Genbank
Lactobacillus mucosae KHPC15	1.9	Cow rumen/NZ	SAMN05216545	BioSample/ Hungate1000*
Lactobacillus mucosae KHPX11	1.9	Cow rumen/NZ	SAMN05216461	BioSample/ Hungate1000*
Lactobacillus mucosae WCC8	1.9	Cow rumen/NZ	SAMN05216430	BioSample/ Hungate1000*
Lactobacillus mucusae AGR63	1.9	Cow rumen/NZ	SAMN02744693	BioSample/ Hungate1000*
Lactobacillus plantarum AG30	3.4	Sheep rumen/NZ	JHWA00000000.1	Genbank
Lactobacillus ruminis DSM 20403	2	Cow rumen/UK	GCF_001436475.1	Genbank
Lactobacillus ruminis WC1T17	1.8	Cow rumen/NZ	SAMN05216431	BioSample/ Hungate1000*
Lactococcus garvieae M79	2.2	Camel forestomach/Australia	SAMN05216438	BioSample/ Hungate1000*
Lactococcus lactis 511	2.5	Cow rumen/NZ	JNLP00000000.1	Genbank
Megamonas sp. Calf98-2	2.2	Calf faeces/NZ	SAMN05216340	BioSample/ Hungate1000*
Megasphaera elsdenii J1	2.5	Sheep rumen/UK	NZ_FRAK00000000 .1	Genbank
Megasphaera elsdenii DSM 20460	2.5	Sheep rumen/UK	NC_015873.1	Genbank
Megasphaera elsdenii YE34	2.4	Cow rumen/Australia	FOQP00000000.1	Genbank
Megasphaera elsdenii T81	2.5	Cow rumen/USA	JHXC00000000.1	Genbank
Methanobrevibacter boviskoreani JH1	2.1	Cow rumen/Korea	GCF_000320505.1	Genbank
Methanobrevibacter gottschalkii DSM 11978	1.9	Pig faeces/USA	SAMN05216439	BioSample/

				Hungate1000*
Methanobrevibacter millerae DSM 16643	2.7	Cow rumen/Australia	SAMN02910315	BioSample/ Hungate1000*
Methanobrevibacter millerae SM9	2.5	Sheep rumen/NZ	GCF_001477655.1	Genbank
Methanobrevibacter olleyae DSM 16632	2.1	Sheep rumen/Australia	FOTL00000000.1	Genbank
Methanobrevibacter olleyae YLM1	2.2	Lamb rumen/NZ	SAMN04335716	BioSample/ Hungate1000*
Methanobrevibacter wolinii SH	2	Sheep faeces/USA	JHWX00000000.1	Genbank
Methanobrevibater ruminantium M1	2.9	Cow rumen/USA	NC_013790.1	Genbank
Methanomicrobium mobile DSM 1539	1.7	Cow rumen/USA	JOMF00000000.1	Genbank
Methanosarcina sp. DSM 11855	3.1	Sheep rumen/Germany	PRJNA254857	BioProject/ Hungate1000*
Micrococcineae bacterium KH10	2.8	Cow rumen/NZ	PRJNA370722	BioProject/ Hungate1000*
Micrococcus luteus VTM4R57	2.6	Moose rumen/USA	NZ_FRCE00000000 .1	Genbank
Mitsuokella jalaludinii DSM 13811	2.4	Cow rumen/Malaysia	JNKR00000000.1	Genbank
Morganella morganii NLAE-zl-C84	3.9	Cow faeces enrichment/USA	SAMN05216301	BioSample/ Hungate1000*
Olsenella sp. KH1P3	2	Cow rumen/NZ	SAMN06298223	BioSample/ Hungate1000*
Olsenella sp. KH2P3	2.4	Cow rumen/NZ	NZ_FPKD00000000 .1	Genbank
Olsenella sp. KH3B4	2.4	Cow rumen/NZ	SAMN05216348	BioSample/ Hungate1000*
Olsenella umbonata DSM 22619	2.2	Sheep rumen/UK	FMZL00000000.1	Genbank
Olsenella umbonata WCP15	2	Cow rumen/NZ	SAMN05216447	BioSample/ Hungate1000*
Oribacterium sp. FC2011	4.2	Cow rumen/NZ	JNJM00000000.1	Genbank

Oribacterium sp. KHPX15	4	Cow rumen/NZ	SAMN05216349	BioSample/ Hungate1000*
Oribacterium sp. NK2B42	3.8	Sheep rumen/NZ	AUJX00000000	Genbank
Oribacterium sp. P6A1	3.7	Cow rumen/NZ	NZ_JNKO00000000.1	Genbank
Oribacterium sp. WCC10	3.6	Cow rumen/NZ	SAMN05216356	BioSample/ Hungate1000*
Oscillibacter sp. PC13	2.8	Sheep rumen/Australia	NZ_FOXE00000000.1	Genbank
Pediococcus acidilactici AGR20	1.9	Sheep rumen/NZ	JAGU00000000.1	Genbank
Peptoclostridium mangenotii LM2	3	Lamb faeces/NZ	JIAA00000000.1	Genbank
Peptostreptococcaceae bacterium pGA-8	1.8	Cow rumen/NZ	FONK00000000.1	Genbank
Peptostreptococcaceae bacterium VA2	3.6	Deer faeces/NZ	JMMB00000000.1	Genbank
Peptostreptococcus anaerobius C	2.1	Cow rumen/USA	PRJNA254856	BioProject/ Hungate1000*
Peptostreptococcus russellii Calf135	2.1	Calf faeces/NZ	SAMN05216454	BioSample/ Hungate1000*
Peptostreptococcus sp. D1	2.3	Deer rumen/NZ	FONP00000000.1	Genbank
Porphyromonadaceae bacterium KH3CP3RA	4.9	Cow rumen/NZ	GCA_900114365.1	Genbank
Porphyromonadaceae bacterium KH3R12	4.2	Cow rumen/NZ	SAMN05216331	BioSample/ Hungate1000*
Porphyromonadaceae bacterium KHP3R9	3.4	Cow rumen/NZ	GCA_900116745.1	Genbank
Porphyromonadaceae bacterium NLAE-zl-C104	3.9	Cow faeces enrichment/USA	SAMN05216365	BioSample/ Hungate1000*
Prauserella rugosa DSM 43194	5.3	Cow rumen/Italy	SAMN02946344	BioSample/ Hungate1000*
Prevotella albensis DSM 11370	2.7	Sheep rumen/UK	SAMN02440641	BioSample/ Hungate1000*

Prevotella brevis ATCC 19188	3.5	Cow rumen/USA	SAMN02744010	BioSample/ Hungate1000*
Prevotella brevis P6B11	3.2	Cow rumen/NZ	JHXG00000000.1	Genbank
Prevotella bryantii B14	3.6	Cow rumen/USA	ADWO0000000.1	Genbank
Prevotella bryantii C21a	3.2	Cow rumen/NZ	AUKF00000000.1	Genbank
Prevotella bryantii FB3001	3.3	Cow rumen/NZ	NZ_FNHC00000000.1	Genbank
Prevotella bryantii KHPX14	3.3	Cow rumen/NZ	SAMN05216455	BioSample/ Hungate1000*
Prevotella ruminicola 23	3.6	Cow rumen/USA	CP002006.1	Genbank
Prevotella ruminicola AR32	3.2	Sheep rumen/Australia	SAMN05216354	BioSample/ Hungate1000*
Prevotella ruminicola BPI-162	3.6	Sheep rumen/Japan	NZ_FOLF00000000.1	Genbank
Prevotella ruminicola BPI-34	3.7	Sheep rumen/Japan	NZ_FRCJ00000000.1	Genbank
Prevotella ruminicola D31d	3.8	Cow rumen/USA	SAMN05216462	BioSample/ Hungate1000*
Prevotella ruminicola KHP1	3.4	Cow rumen/NZ	SAMN04487826	BioSample/ Hungate1000*
Prevotella ruminicola KHT3	4	Cow rumen/NZ	SAMN05216463	BioSample/ Hungate1000*
Prevotella ruminicola RM4	3.4	Cow rumen/Japan	JNKG00000000.1	Genbank
Prevotella ruminocola Ga6B6	3.5	Cow rumen/NZ	JHXD00000000.1	Genbank
Prevotella sp. AGR2160	2.9	Calf faeces/NZ	AUJK00000000.1	Genbank
Prevotella sp. BPI-145	3.6	Sheep rumen/Japan	NZ_FNIW00000000.1	Genbank
Prevotella sp. BPI-148	3.5	Sheep rumen/Japan	NZ_FNCQ00000000.1	Genbank

Prevotella sp. FD3004	3	Cow rumen/NZ	JQKR00000000.1	Genbank
Prevotella sp. HUN102	3.3	Cow rumen/NZ	JIAF00000000.1	Genbank
Prevotella sp. KH1P2	3.3	Cow rumen/NZ	FOHY00000000.1	Genbank
Prevotella sp. KH2C16	3.2	Cow rumen/NZ	SAMN05216383	BioSample/ Hungate1000*
Prevotella sp. KHP7	3.1	Cow rumen/NZ	FOIV00000000.1	Genbank
Prevotella sp. LC2012	2.9	Cow rumen/NZ	FNUF00000000.1	Genbank
Prevotella sp. MA2016	3.4	Cow rumen/NZ	JHUW00000000.1	Genbank
Prevotella sp. NE3005	3.6	Cow rumen/NZ	NZ_FOCK00000000 .1	Genbank
Prevotella sp. P6B1	3.6	Cow rumen/NZ	JNKF00000000.1	Genbank
Prevotella sp. P6B4	3.6	Cow rumen/NZ	JHVE00000000.1	Genbank
Prevotella sp. TC2-24	2.9	Cow rumen/Sweden	SAMN04487850	BioSample/ Hungate1000*
Prevotella sp. TC2-28	3.4	Cow rumen/Sweden	NZ_FNRE00000000 .1	Genbank
Prevotella sp. TF2-5	3.5	Cow rumen/Sweden	NZ_FOWK0000000 0.1	Genbank
Prevotellaceae bacterium HUN156	2.8	Cow rumen/NZ	NZ_FPIT00000000. 1	Genbank
Prevotellaceae bacterium KH2P17	3.3	Cow rumen/NZ	PRJNA370726	BioProject/ Hungate1000*
Propionibacteriaceae bacterium P6A17	2.7	Cow rumen/NZ	JHVD00000000.1	Genbank
Propionibacterium sp. MB3007	2.5	Cow rumen/NZ	JMLB00000000.1	Genbank
Proteiniclasticum ruminis DSM 24773	3.1	Yak rumen/China	JNKC00000000.1	Genbank
Proteus mirabilis NLAE-zl-C285	3.8	Cow faeces enrichment/USA	SAMN05216484	BioSample/ Hungate1000*
Proteus mirabilis NLAE-zl-G534	4	Goat faeces enrichment/USA	NZ_FOPN00000000 .1	Genbank

Pseudobutyrivibrio ruminis ACV-9	2.8	Cow rumen/NZ	SAMN02910377	BioSample/ Hungate1000*
Pseudobutyrivibrio ruminis AD2017	2.9	Cow rumen/NZ	AUJQ00000000	Genbank
Pseudobutyrivibrio ruminis CF1b	3.3	Sheep caecum/USA	SAMN02440829	BioSample/ Hungate1000*
Pseudobutyrivibrio ruminis DSM 9787	3	Cow rumen/Sweden	SAMN02910411	BioSample/ Hungate1000*
Pseudobutyrivibrio ruminis HUN009	2.8	Cow rumen/NZ	JNLH00000000.1	Genbank
Pseudobutyrivibrio sp. 49	3.4	Cow rumen/USA	SAMN05421493	BioSample/ Hungate1000*
Pseudobutyrivibrio sp. ACV-2	2.7	Cow rumen/NZ	FNQZ00000000.1	Genbank
Pseudobutyrivibrio sp. AR14	3.1	Sheep rumen/Australia	PRJNA254864	BioProject/ Hungate1000*
Pseudobutyrivibrio sp. C4	2.9	Cow rumen/NZ	SAMN029104	BioSample/ Hungate1000*
Pseudobutyrivibrio sp. JW11	3.1	Sheep rumen/UK	NZ_FOWB00000000.1	Genbank
Pseudobutyrivibrio sp. LB2011	2.8	Cow rumen/NZ	GCA_000701765.1	Genbank
Pseudobutyrivibrio sp. MD2005	3.1	Cow rumen/NZ	JHXE00000000.1	Genbank
Pseudobutyrivibrio sp. NOR37	3	Cow rumen/UK	FOZB00000000.1	Genbank
Pseudobutyrivibrio sp. OR37	3.6	Cow rumen/Canada	PRJNA311407	BioProject/ Hungate1000*
Pseudobutyrivibrio sp. UC12254	3.4	Cow rumen/USA	FOVQ00000000.1	Genbank
Pseudobutyrivibrio sp. YE44	3.2	Cow rumen/Australia	SAMN02910298	BioSample/ Hungate1000*
Pseudobutyrivibrio xylanivorans DSM 10317	3.2	Sheep rumen/South Africa	SAMN02910350	BioSample/ Hungate1000*
Ruaniaceae bacterium KH17	2.7	Cow rumen/NZ	NZ_FZQV00000000.1	Genbank

Ruminobacter amylophilus DSM 1361	2.8	Sheep rumen/UK	SAMN02910344	BioSample/ Hungate1000*
Ruminobacter amylophilus RM87	2.9	Cow rumen/Japan	JNKD00000000.1	Genbank
Ruminococcacea bacterium YAD3003	2.7	Cow rumen/NZ	SAMN02910264	BioSample/ Hungate1000*
Ruminococcaceae bacterium AB4001	3.2	Cow rumen/NZ	JHXJ00000000.1	Genbank
Ruminococcaceae bacterium AE2021	3.1	Cow rumen/NZ	JIAE00000000.1	Genbank
Ruminococcaceae bacterium D5	2.6	Deer rumen/NZ	FORK00000000.1	Genbank
Ruminococcaceae bacterium FB2012	3.8	Cow rumen/NZ	SAMN02910317	BioSample/ Hungate1000*
Ruminococcaceae bacterium KHP2	3	Cow rumen/NZ	PRJNA370723	BioProject/ Hungate1000*
Ruminococcaceae bacterium NK3B98	3	Sheep rumen/NZ	ATWA00000000.1	Genbank
Ruminococcaceae bacterium P7	3.1	Cow rumen/Japan	FMUA00000000.1	Genbank
Ruminococcaceae bacterium YRB3002	3	Cow rumen/NZ	SAMN02910456	BioSample/ Hungate1000*
Ruminococcus albus 7	4.5	Cow rumen/USA	PRJNA42255	BioProject/ Hungate1000*
Ruminococcus albus 8	4.1	Cow rumen/USA	ADKM00000000	Genbank
Ruminococcus albus AD2013	4.2	Cow rumen/NZ	JAGS00000000.1	Genbank
Ruminococcus albus AR67	4.3	Sheep rumen/Australia	SAMN02910406	BioSample/ Hungate1000*
Ruminococcus albus KH2T6	3.8	Cow rumen/NZ	SAMN05216469	BioSample/ Hungate1000*
Ruminococcus albus SY3	4.3	Sheep rumen/UK	JEOB00000000.1	Genbank
Ruminococcus bromii YE282	2.5	Cow rumen/Australia	FMUV00000000.1	Genbank
Ruminococcus flavefaciens 17	3.5	Cow rumen/UK	AFNE00000000	Genbank
Ruminococcus flavefaciens AE3010	3.7	Cow rumen/NZ	JAGT00000000.1	Genbank
Ruminococcus flavefaciens ATCC 19208	3.6	Cow rumen/USA	KI912489.1	Genbank

Ruminococcus flavefaciens FD-1	4.6	Cow rumen/USA	ACOK00000000	Genbank
Ruminococcus flavefaciens MA2007	3.4	Cow rumen/NZ	JHXF00000000.1	Genbank
Ruminococcus flavefaciens MC2020	3.1	Cow rumen/NZ	PRJNA223444	BioProject/ Hungate1000*
Ruminococcus flavefaciens ND2009	3.6	Cow rumen/NZ	JHXI00000000.1	Genbank
Ruminococcus flavefaciens SAb67	3.8	Sheep abomasum/NZ	PRJNA254877	BioProject/ Hungate1000*
Ruminococcus flavefaciens XPD3002	3.7	Cow rumen/NZ	NZ_FPJT00000000.1	Genbank
Ruminococcus flavefaciens Y1	3.7	Sheep rumen/Australia	NZ_FRCT00000000.1	Genbank
Ruminococcus flavefaciens YAD2003	3.7	Cow rumen/NZ	SAMN02910265	BioSample/ Hungate1000*
Ruminococcus flavefaciens YL228	3.4	Lamb rumen/NZ	SAMN02910280	BioSample/ Hungate1000*
Ruminococcus flavefaciens YRD2003	3.6	Cow rumen/NZ	NZ_FNZT00000000.1	Genbank
Ruminococcus gnavus AGR2154	3.7	Calf faeces/NZ	JAGQ00000000.1	Genbank
Ruminococcus sp. FC2018	2.4	Cow rumen/NZ	JHXH00000000.1	Genbank
Ruminococcus sp. HUN007	4.2	Cow rumen/NZ	JOOA00000000.1	Genbank
Ruminococcus sp. NK3A76	3.6	Sheep rumen/NZ	JMMA00000000.1	Genbank
Ruminococcus sp. YE71	4.1	Sheep rumen/Australia	SAMN02910447	BioSample/ Hungate1000*
Ruminococcus sp. YE78	4.1	Sheep rumen/Australia	SAMN02910446	BioSample/ Hungate1000*
Sarcina sp. DSM 11001	4.2	Cow rumen/Sweden	FNFZ00000000.1	Genbank
Selenomonas bovis 8-14-1	2.6	Cow rumen/Japan	JNKB00000000.1	Genbank
Selenomonas bovis DSM 23594	2.7	Yak rumen/China	NZ_ARLB00000000.1	Genbank

Selenomonas ruminantium AB3002	3.7	Cow rumen/NZ	JNIO00000000.1	Genbank
Selenomonas ruminantium AC2024	2.8	Cow rumen/NZ	NZ_JIAC00000000.1	Genbank
Selenomonas ruminantium C3	2.8	Cow rumen/NZ	SAMN02910323	BioSample/ Hungate1000*
Selenomonas ruminantium DSM 2872	3	Cow rumen/USA	PRJNA254915	BioProject/ Hungate1000*
Selenomonas ruminantium GACV-9	2.8	Cow rumen/NZ	SAMN02910356	BioSample/ Hungate1000*
Selenomonas ruminantium HD4	3.1	Cow rumen/USA	SAMN05216582	BioSample/ Hungate1000*
Selenomonas ruminantium KH1T6	3.6	Cow rumen/NZ	SAMN05216583	BioSample/ Hungate1000*
Selenomonas ruminantium L14	3.3	Camel forestomach/Australia	SAMN05216587	BioSample/ Hungate1000*
Selenomonas ruminantium S137	3.2	Sheep rumen/Japan	NZ_FNJQ00000000.1	Genbank
Selenomonas ruminantium TAM6421	3.6	Sheep rumen/Japan	PRJNA60235	BioProject/ Hungate1000*
Selenomonas ruminantium WCT3	2.7	Cow rumen/NZ	SAMN05216584	BioSample/ Hungate1000*
Selenomonas ruminantium Z108	3.1	Sheep rumen/UK	NZ_FOQK00000000.1	Genbank
Selenomonas ruminatium ATCC 12561	3.4	Cow rumen/USA	AUJE00000000	Genbank
Selenomonas sp. AE3005	2.8	Cow rumen/NZ	JMME00000000.1	Genbank
Selenomonas sp. FC4001	2.9	Cow rumen/NZ	JNJF00000000.1	Genbank
Selenomonas sp. ND2010	2.9	Cow rumen/NZ	JNKI00000000.1	Genbank
Sharpea azabuenensis DSM 20406	2.4	Calf rumen/USA	SAMN04487834	BioSample/ Hungate1000*
Sharpea azabuensis DSM 18934	2.4	Horse faeces/Japan	JNKU00000000.1	Genbank

Sharpea azabuensis KH1P5	2.3	Cow rumen/NZ	SAMN04487835	BioSample/ Hungate1000*
Sharpea azabuensis KH2P10	2.3	Cow rumen/NZ	FOMV00000000.1	Genbank
Shigella sonnei NLAE-zl-G496	4.9	Goat faeces enrichment/USA	NZ_FNIS00000000.1	Genbank
Slackia heliotrinireducens DSM 20476	3.2	Sheep rumen/Australia	NC_013165.1	Genbank
Staphylococcus epidermidis AG42	2.6	Sheep rumen/UK	JNLI00000000.1	Genbank
Staphylococcus epidermidis NLAE-zl-G239	2.5	Goat faeces enrichment/USA	NZ_FOPD00000000.1	Genbank
Streptococcus equinus 2B	1.9	Sheep rumen/UK	JNKQ00000000.1	Genbank
Streptococcus equinus AG46	1.9	Sheep rumen/NZ	SAMN02841210	BioSample/ Hungate1000*
Streptococcus equinus AR3	2	Sheep rumen/Australia	SAMN05216470	BioSample/ Hungate1000*
Streptococcus equinus B315	1.8	Sheep rumen/NZ	AUJD00000000.1	Genbank
Streptococcus equinus C277	1.8	Sheep rumen/UK	NZ_FNKC00000000.1	Genbank
Streptococcus equinus ES1	1.8	Sheep rumen/UK	NZ_FODM00000000.1	Genbank
Streptococcus equinus GA-1	1.8	Cow rumen/NZ	SAMN02910449	BioSample/ Hungate1000*
Streptococcus equinus H24	1.9	Calf rumen/USA	SAMN05216373	BioSample/ Hungate1000*
Streptococcus equinus HC5	1.9	Cow rumen/USA	JPGC00000000.1	Genbank
Streptococcus equinus JB1	2	Cow rumen/USA	AUZH00000000.1	Genbank
Streptococcus equinus MPR1	1.9	Camel forestomach/Australia	SAMN05216477	BioSample/ Hungate1000*
Streptococcus equinus MPR2	1.9	Camel forestomach/Australia	SAMN05216384	BioSample/ Hungate1000*

Streptococcus equinus MPR4	1.9	Camel forestomach/Australia	SAMN05216385	BioSample/ Hungate1000*
Streptococcus equinus pGA-7	1.8	Cow rumen/NZ	NZ_FNJX00000000.1	Genbank
Streptococcus equinus pR-5	1.8	Cow rumen/NZ	NZ_FNZW00000000.1	Genbank
Streptococcus equinus Sb04	1.9	Cow rumen/Australia	SAMN05216347	BioSample/ Hungate1000*
Streptococcus equinus Sb05	2	Cow rumen/Australia	SAMN05216392	BioSample/ Hungate1000*
Streptococcus equinus Sb09	2	Cow rumen/Australia	SAMN05216400	BioSample/ Hungate1000*
Streptococcus equinus Sb10	1.9	Cow rumen/Australia	SAMN05216407	BioSample/ Hungate1000*
Streptococcus equinus Sb13	1.9	Cow rumen/Australia	SAMN05216408	BioSample/ Hungate1000*
Streptococcus equinus Sb17	1.9	Cow rumen/Australia	SAMN05216415	BioSample/ Hungate1000*
Streptococcus equinus Sb18	2	Cow rumen/Australia	SAMN05216346	BioSample/ Hungate1000*
Streptococcus equinus Sb20	1.9	Cow rumen/Australia	SAMN05216416	BioSample/ Hungate1000*
Streptococcus equinus SI	1.9	Sheep rumen/Australia	SAMN05216422	BioSample/ Hungate1000*
Streptococcus equinus SN033	1.8	Deer rumen/NZ	ATWZ00000000.1	Genbank
Streptococcus equinus YE01	1.9	Goat rumen/Australia	SAMN05216423	BioSample/ Hungate1000*
Streptococcus gallolyticus LMG 15572	2.4	Goat rumen/Australia	FPBN00000000.1	Genbank
Streptococcus gallolyticus VTM1R27	2.2	Moose rumen/USA	SAMN02910295	BioSample/ Hungate1000*

Streptococcus gallolyticus VTM1R29	2.2	Moose rumen/USA	SAMN04487839	BioSample/ Hungate1000*
Streptococcus gallolyticus VTM2R47	2.2	Moose rumen/USA	NZ_FOGM00000000.1	Genbank
Streptococcus gallolyticus VTM3R24	2.2	Moose rumen/USA	SAMN04487841	BioSample/ Hungate1000*
Streptococcus gallolyticus VTM3R42	2.2	Moose rumen/USA	NZ_FNFJ00000000.1	Genbank
Streptococcus henryi A-4	2.4	Cow rumen/NZ	SAMN02910293	BioSample/ Hungate1000*
Streptococcus sp. 45	1.9	Camel forestomach/Australia	SAMN05216460	BioSample/ Hungate1000*
Streptococcus sp. NLAE-zl-C503	1.9	Cow faeces enrichment/USA	SAMN05216494	BioSample/ Hungate1000*
Succiniclasticum ruminis DSM 11005	2.9	Cow rumen/Sweden	NZ_FMYW00000000.1	Genbank
Succiniclasticum ruminis DSM 9236	2.5	Cow rumen/Sweden	SAMN05216245	BioSample/ Hungate1000*
Succinivibrio dextrinosolvens 22B	3.1	Sheep rumen/UK	NZ_FOSF00000000.1	Genbank
Succinivibrio dextrinosolvens ACV-10	3	Cow rumen/NZ	FPAH00000000.1	Genbank
Succinivibrio dextrinosolvens H5	2.7	Sheep rumen/NZ	JNKL00000000.1	Genbank
Terrisporobacter glycolicus KPPR-9	4	Cow rumen/NZ	SAMN02910355	BioSample/ Hungate1000*
Treponema bryantii B25	3.4	Cow rumen/USA	SAMN04487977	BioSample/ Hungate1000*
Treponema bryantii NK4A124	3.3	Sheep rumen/NZ	ATWV00000000.1	Genbank
Treponema bryantii XBD1002	3.2	Cow rumen/NZ	FORI00000000.1	Genbank
Treponema saccharophilum DSM 2985	3.5	Cow rumen/USA	AGRW00000000.1	Genbank
Treponema sp. C6A8	2.9	Cow rumen/NZ	JHVB00000000.1	Genbank

Wolinella succinogenes DSM 1740	2.1	Cow rumen/USA	BX571656.1	Genbank

*: genomes obtained from the Genome portal in the JGI genome database (The Hungate1000 Project).

Table 3. Metatranscriptome datasets used in this study (data collected in August 2018).

SRA Study	Sample name	Spots (M)	Bases (Gbp)	Size (G)	GC content (%)	Published	Experiment	Biosample	Organism
SRR769401	human	6.3	1.3	1.1	50.8%	08.03.2013	SRX247334	SAMN01915224 (SRS399315)	human gut
SRP053865	beef rumen	29.5	5.9	4	47.9%	01.03.2017	SRX1647089	SAMN04569317 (SRS1350934)	bovine gut
SRP111123	dairy rumen	62.9	15.7	5.2	48.9%	31.12.2017	SRX2984721	SAMN07313972 (SRS2337472)	bovine gut

GENERAL CONCLUSIONS

- The rumen microbiome represents a reservoir of ARG's and shows enriched abundance of tetracycline resistance genes;

- Resfinder, Resfams and ARG-ANNOT differ in their capacity to identify ARGs in microbial genomes;

- A novel ICE structure harboring the *tet*(W) gene, named in this study as ICE_*RbtetW*_07, has been identified in different species of ruminal bacteria, indicating the acquision and potential to spread of this gene by horizontal gene transfer events;

- The *tet*(W) gene in ruminal bacterial genomes is under positive selective pressure;

- ARGs found in ruminal bacterial genomes are expressed both in the host and in the human gastrointestinal tract (GIT);

- The *in vitro* analysis confirmed some of the resistance phenotypes predicted *in silico*, including the vancomycin resistante in the ruminal bacteria *Blautia schinkii*, where our *in silico* analysis have identified a complete operon of vancomycin resistance not yet described in this microorganism.