Host genetics affected the resistome and its expression patterns in the rumen of beef cattle raised without antibiotics used in humans

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Research

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Abstract

Background: The rumen microbiome is a potential reservoir of antimicrobial-resistant genes (ARGs), termed resistome. However, the activity of ARGs and what factors affect expression of ARGs in the rumen is unknown. Here, the rumen resistome was evaluated using metagenomic and metatranscriptomic datasets, with the aim to identify the active rumen resistome and whether it can be affected by cattle breed and feed efficiency.

Results: Genes encoding resistance to 12 ARG classes representing 62 individual ARGs were detected in the rumen metagenomes of Angus, Charolais or Kinsella composite hybrid (KC) beef steers (n = 48) with high and low feed efficiency. Three genes encoding tetracycline (*tetQ*, *tetW*) and macrolide (*mefA*) resistance constituted 75.3% of abundance of total ARGs identified in all animals, suggesting they are 'core' resistome in the rumen of steers. Only about 20.96% (13/62) of the total ARGs identified were expressed, among which genes encoding resistance to tetracycline, macrolide-licosamide-streptogramin (MLS), aminoglycoside, and multidrug exhibited the highest level of expression. More than half (56.2%) of the ARGs identified were plasmid-associated, while only 5 plasmid-associated ARGs were expressed. The abundance of 17, 14, and 5 individual ARGs were significantly affected by breed, feed efficiency, and breed × feed efficiency, respectively, while the expression of ARGs did not differ among breeds or between feed efficiency groups. In KC cattle, less number of total ARGs, ARG transcripts, as well as total active bacteria (estimated by 16S rRNA copies) was observed than AN. The total active bacteria were negatively correlated with expression of MLS and tetracycline ARG (*mefA*, *tet40*, *tetM*, *tetW*, and an unidentified *tet*), and tended to be negatively correlated with the expression of plasmid-associated tetracycline ARG t only in the rumen of KC cattle.

Conclusions: Our results suggest that a large portion of the ARGs are not expressed in the rumen of cattle raised without antibiotics used in humans. The identified less diversified active resistome and total active bacteria, and the significant correlation between total active bacteria and the abundance of ARG transcripts in KC cattle suggest that the expression of resistome in the rumen may be breed specific and driven by ruminal microbiota.

Background

Antimicrobials have been widely used in food producing animals since 1950th to enhance feed efficiency, accelerate growth, and minimize disease [1]. It is estimated that antimicrobials used to prevent/treat disease and/or promote growth in chickens, pigs, and cattle will increase from 63,151 tonnes in 2010 to 105,596 tonnes in 2030 [2]. Consumption of antimicrobials in livestock, as well as antimicrobial residues in food have been proposed to contribute to antimicrobial resistance (AMR) in humans [3, 4] and aquatic/soil environments [5, 6]. The AMR found in food-producing animals not only reduces the therapeutic efficacy of antimicrobials against diseases but also selects for reservoirs of resistance that could be transferred to humans via the food chain or through the environment [7]. Therefore, reducing

antimicrobial resistance and preventing antimicrobial residues from entering the food chain is a priority for livestock industry to address the food safety and public health concerns [8].

In fact, AMR in bacteria is an ancient phenomenon and was present long before the widespread clinical and agricultural use of antimicrobials [9]. Numerous antimicrobial resistance genes (ARGs) encode for resistance to an array of antimicrobials [9] that bacteria produce to compete and survive within complex ecological systems [10]. Recent efforts have documented the rumen resistome, a vast reservoir of ARGs that could be acquired by human commensals and pathogens [11] in beef cattle [12–14], dairy cattle [14], and sheep [15], and revealed that ARGs can be affected by diet [13] or antibiotic treatment [12]. However, these studies only assessed ARGs at genomic level using metagenomics based on short-read sequencing [12, 13, 15] or long-read sequencing [14] and with a limited number of animals were involved. On the other hand, gene expression is a better proxy for assessing functional activity within biological ecosystems [16]. It is largely unknown the extent to which ARGs are expressed in the rumen of cattle that are not under the selective pressure antibiotics used in human is unclear. Recent studies have assessed wastewater treatment plant resistome at both metagenomic and metatranscriptome levels and reveals that plant locations not only affected the ARGs but also their transcripts [7, 17]. However, comparing to largely identified resistomes in the animal system, their functionality and activity *in vivo* has not been reported.

More and more evidences have revealed the individualized rumen microbiome when animals are fed the same diet and host genetic factors have been reported to drive pan microbiome [18]. We hypothesized that 1) rumen resistome, similar as rumen microbiome can be affected by cattle genetics such as breed and/or feed efficiency; 2) not all ARGs are expressed and the expression of ARGs, including plasmid-associated ARGs, can be affected by active microbiome and/or cattle genetics; 3) as rumen microbiome differs in animals between high and low feed efficiency, and less diverse active rumen microbiome was associated with higher feed efficiency in cattle [19], we also hypothesized that animals selected with high feed efficiency may have higher expression of ARGs because lower bacterial diversity is associated with enriched resistome in human taken antibiotics [20]. Therefore, in this study we assessed the presence and expression of ARGs in the rumen of beef steer differing in breed and feed efficiency raised without antibiotics used in human medicine. Understanding of the expression of ARGs in the rumen could provide important insights into resistome function and if factors such as host genetics or antibiotic usage selective pressures play a role in AMR function *in vivo*.

Methods

Animal experiment and sample collection

The datasets used in the current study were part from our previous study by Li et al. [21]. In brief, ruminal digesta samples were collected from 48 steers, consisting of three breeds and two residual feed intake (RFI) groups (Kinsella composite hybrid [KC, H-RFI, n = 8; L-RFI = 8]; Angus [AN, H-RFI, n = 8; L-RFI = 8], and Charolais [CH, H-RFI, n = 8; L-RFI = 8]). H-RFI, which was 1.45 ± 0.17 kg of body weight/day, was

considered as inefficient while L-RFI, which was -1.64 ± 0.21 kg of body weight/day, was considered as efficient as described by Li et al. [21]. All steers were raised in the same feedlot condition and fed the same diet that consisted of 80% barley grain, 15% barley silage, and 5% supplement. The supplement contained 33 ppm monensin, an antibiotic that is not used in human medicine.

Metagenome and metatranscriptome sequencing

Rumen digesta was collected at slaughter and snap-frozen in liquid nitrogen as described by Li et al. [21]. Total metagenomic DNA and RNA were isolated from rumen digesta using the methods of Yu and Morrison [22] and Li et al. [23], respectively. After quality and quantity checks, metagenomic libraries of the DNA were constructed using TruSeq DNA PCR-Free Library Preparation Kit (Illumina, San Diego, CA, USA) and subjected to Illumina (HiSeq 2000) sequencing. After measurement of RNA yield and quality, samples with RNA integrity number (RIN) > 7.0 were used to construct metatranscriptome libraries using the TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA). All metagenomic and metatranscriptomic libraries were sequenced at the McGill University and Génome Québec Innovation Centre (Montréal, QC, Canada) using an Illumina HiSeq 2000 platform (100 bp paired-end sequencing of ~ 350 bp inserts for metagenome, and of ~ 140 bp inserts for metatranscriptome). Quality control (QC) of each dataset was performed as described by Li et al. [21].

Detection of ARGs and plasmid-associated ARGs

The prevalence and abundance of the rumen resistome profile of each metagenomic dataset was determined using a two-stage (ARG-OAP 2.0) pipeline [24]. Briefly, post quality-controlled reads (paired-end) from each sample were blasted against the Structured ARG database (SARG), comprised of the Antibiotic Resistance Genes Database (ARDB) and the Comprehensive Antibiotic Resistance Database (CARD), to extract ARG-like reads. Those reads were subsequently annotated as ARG-like reads at the cut-off of E value of 10^{-7} , sequence identity of 80% and alignment length more than > 25 amino acids. By using this cut-off, the identification accuracy can reach up to 99.5% [25].

Plasmid-associated ARG was determined using a modified ARG-OAP 2.0 pipeline. Instead of SARG, reads were blasted against the ACLAME database, a database for identifying mobile genetic elements [26], with a cut-off E value of $\leq 10^{-7}$ criteria with amino acid identity $\geq 80\%$ and coverage $\geq 70\%$.

Abundance of ARGs and ARG transcripts

The abundance of ARG classes, total ARGs, and individual ARG were calculated with normalization for the sequence length and the number of 16S rRNA genes, as well as the ARG reference sequence length according to Yin et al. [24], which was defined as 'copy of ARG per copy of 16S-rRNA gene', following the most recent approaches in the literature [7, 27].

To evaluate the expressions of resistome, the extracted ARG-like sequences identified in metagenomic datasets were used as a reference database to support extraction of ARG-like transcripts from the metatranscriptome datasets using ARG-OAP 2.0 pipeline. Reads were annotated as ARG-like transcripts using the same cutoffs as described above. The abundance of ARG classes transcript, total ARG transcripts, and individual ARG transcript was reported as 'ppm' (number of ARGs sequences in one million sequences) following a method reported by Yin et al. [24].

Estimation of active rumen microbiome using qPCR

Total RNA (1 µg) was reversely transcribed using iScript reverse transcription Supermix for quantitative real-time PCR kit (qRT-PCR; Bio-Rad Laboratories, Hercules, CA) to generate complementary DNA (cDNA). The 20 times-diluted cDNAs were used to evaluate the abundances of total active bacteria by measuring its copy number of 16S rRNA using qPCR with bacterial universal primers bacteria (U2-F: ACTCCTACGGGAGGCAG; U2-R: GACTACCAGGGTATCTAATCC) [28]. The qPCR was performed using SYBR Green chemistry with StepOnePlus Real-Time PCR System (Applied Biosystems) and the programs were as follows: for bacteria, the holding stage at 95 °C for 5 min, followed by 40 cycles at 95 °C for 20 s and 60 °C for 30 s. Standard curves were constructed using serial dilutions of purified plasmid containing full length 16S rRNA gene of *Butyrivibrio hungatei* for total bacteria. The copy number of 16S rRNA of total bacteria/g rumen contents was calculated with an equation previously described by Malmuthuge et al. [29].

Statistical analyses

The cut-off for detected ARG class or individual ARG was abundance > 0 in at least half samples for one breed. Variation in the number (prevalence) and abundance of ARGs and ARG transcripts among breeds, between H- and L-RFI, as well as their interactions (breed × feed efficiency), were analysed using the aligned rank transform (ART) method [30], using 'ARTool' package in R (version 3.6.1). The ART analysis was only conducted when an ARG class/ARG class transcript or individual ARG/ARG transcript was detected in more than 3 samples in all three breeds. The *P*-value of multiple comparison of breed effect was adjusted into false discovery rate (FDR) using the Benjamini-Hochberg algorithm using 'dunn_test' in R (version 3.6.1). Circos plot analysis was performed in R using the RCircos package [31]. Principle component analysis (PCA) of ARGs and ARG transcripts was conducted using multivariate analysis of variance (MANOVA). Spearman correlation analysis was performed for the abundance of expressed ARGs and log-transformed total active bacteria copy numbers and the results were visualized using 'ggscatter' in R (version 3.6.1). The Spearman's correlation coefficient, known as rho (ρ), ranges from -1.00 (a perfect negative correlation) to +1.00 (a perfect positive correlation). Significant difference was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. For Spearman correlation, a rho value between 0.40 and 0.65 and P value ≤ 0.05 is considered as significant correlation, while P value between 0.05 and 0.10 is considered as tendency towards correlation.

Results

ARG profiles detected in the rumen microbiome of beef steers

On average, the ARG-like reads accounted for $0.10\% \pm 0.003\%$ (SD) of the metagenomic reads in the rumen of 48 beef steers. In total, 12 ARG classes were detected with genes encoding resistance to aminoglycoside, bacitracin, macrolide-lincosamide-streptogramin (MLS), multidrug resistance (MDR), tetracycline, and vancomycin detected in all 48 rumen samples, and those encoding resistance to sulfonamide (46/48), beta-lactam (46/48), rifamycin (35/48), fosfomycin (32/48), as well as chloramphenicol (31/48), present in majority of the samples (Additional file 1: Table S1). Tetracycline, followed by MLS and aminoglycoside were the most abundant ARG classes detected (Fig. 1a and 1b).

A total of 62 individual ARGs were detected based on the same cut-off mentioned above, among which 10 genes belonging to tetracycline (e.g., *tet32*, *tet35*, and *tet37*), 6 genes belonging to MLS (e.g., *ermB*, *ermG*, and *lnuA*), 3 genes belonging to aminoglycoside (*aadA*, *aadE*, *ant(9)-I*) and vancomycin (*vanG*, *vanR*, *vanS*), as well as *bacA* belonging to bacitracin and an ABC transporter belonging to MDR were detected in all 48 rumen samples (Additional file 1: Table S1).

Expression of ARGs in the rumen microbiome

Transcripts of genes encoding resistance to aminoglycoside, bacitracin, beta-lactam, MLS, multidrug (MDR), polymyxin, sulfonamide, tetracycline, and vancomycin were detected (Fig. 1c and 1d). In specific, genes encoding resistance to MLS and tetracycline were expressed in all rumen samples, followed by aminoglycoside (37/48), MDR (28/48), vancomycin (19/48), and bacitracin (16/48). A total of 13 of the 62 ARGs showed transcriptional activity (Additional file 1: Table S2). Among these, the transcripts of *mefA*, *tet40*, *tetM*, *tetQ*, an unclassified *tet* gene, and *tetW* were detected in all 48 rumen samples, followed by *tetO* (34/48), *vatB* (28/48), *aadA* (24/48), *ant(9)-I* (23/48), *tet35* (19/48), and *bacA* (16/48).

Comparison of class of ARGs and ARG expression profiles among breeds

Sixty-two ARGs belonged to 12 ARG classes were detected in all three breeds, with the exception of genes encoding fosfomycin resistance which were only found in the rumen of 18.7% (3/16) KC animals (Additional file 1: Table S1). Seven of 12 ARG classes were detected with transcriptional activity in all three breeds (Additional file 1: Table S2). Principle component analysis (PCA) showed no separation of the abundance of ARG classes (Fig. 2a) or the expressed ARG class among breeds (Fig. 2b).

The number of total ARGs detected was lower in KC than in AN (53.2 vs 60.0, $P = 0.026$) and CH (53.2 vs 63.7, $P < 0.001$) (Fig. 3a). The number of ARGs belonging to MLS ($P = 0.003$; Fig. 3b), MDR ($P = 0.010$;

Fig. 3c), and vancomycin ($P=0.004$; Fig. 3d) was also lower in KC than CH. The number of ARGs belonging to MLS was also lower ($P=0.003$, Fig. 3b) in KC than AN. The number of total ARG transcripts (12.2 vs 15.8, $P=0.012$; Fig. 4a), and the number of tetracycline ARG transcripts (6.5 vs 7.8, $P=0.033$; Fig. 4b) was lower in KC than in AN.

The abundance of total ARG was higher in KC ($P=0.022$) and CH ($P=0.002$) than in AN, with no difference between KC and CH ($P=0.684$) (Fig. S1a; Additional file 1: Table S1). The abundance of aminoglycoside resistant genes was higher in KC than AN ($P=0.035$) (Fig. S1b) while that of fosfomycin resistant genes was lower in KC than AN and CH ($P<0.001$) (Fig. S1c). The abundance of MLS ($P=0.010$) (Fig. S1d) and MDR ($P=0.006$) (Fig. S1e) resistant genes was higher in CH than AN whereas that of tetracycline (Fig. S1f) resistant genes was lower in AN than KC ($P=0.028$) and CH ($P=0.017$). At transcripts level, no difference in abundance of any expressed ARG class or individual ARG was detected among breeds (Additional file 1: Table S2).

Differential abundant individual ARGs and ARG transcripts among breeds

The abundance of 17 ARGs were significantly affected by breed. Specifically, the abundance of *aac(6')-I* ($P=0.025$), *aacA-aphD* ($P=0.001$), *mphB* ($P=0.003$), an multidrug ABC transporter ($P=0.023$), *vatB* ($P<0.001$), *vanW* ($P=0.047$) was higher, *ant(9)-I* ($P=0.055$) tended to be higher, while that of *ermA* ($P=0.012$), *ermB* ($P=0.015$), *EmrB/QacA* ($P=0.002$), major_facilitator_superfamily_transporter ($P=0.013$) were lower, and *InuA* tended to be lower ($P=0.085$) in KC than in AN (Fig. 5; Additional file 1: Table S1). Compared to AN, the abundance of class A beta-lactamase ($P=0.009$), *marR* ($P=0.004$), *vanX* ($P=0.012$), *bcrA* ($P=0.017$), *mefA* ($P=0.011$), *vanW* ($P=0.006$), *vatB* ($P=0.001$) was higher, *tet37* ($P=0.056$) tended to be higher, while that of *ermF* ($P=0.049$) and *tetX* ($P=0.026$) was lower in CH. In addition, the abundance of *aacA-aphD* ($P=0.001$), *vanX* ($P=0.001$), *bcrA* ($P<0.001$), *tet37* ($P=0.032$), *InuA* ($P=0.002$), *ermB* ($P=0.012$), a MDR transporter ($P<0.001$), *ermG* ($P=0.024$) was higher, while that of *tetX* ($P=0.002$), *ant(9)-I* ($P=0.024$), class A beta-lactamase ($P<0.001$), *mphB* ($P<0.001$), *EmrB/QacA* ($P=0.001$), major_facilitator_superfamily_transporter ($P<0.001$), *norA* ($P=0.019$) was lower, and *tet44* tended to be lower ($P=0.089$) in KC than in CH.

At transcripts level, no difference in the abundance of any expressed ARG class or individual ARG was detected among breeds (Additional file 1: Table S2).

Differential ARGs and ARG transcripts between RFI

The 12 ARG classes were also detected in both H-RFI and L-RFI beef steers, with the exception that genes encoding polymyxin resistance were only found in the rumen of 33.3% (8/24) of H-RFI animals (Additional file 1: Table S1). Seven of 12 ARG classes were detected with transcriptional activity in both RFI groups (Additional file 1: Table S2). Principle component analysis (PCA) showed no separation of the

abundance of ARG classes (Fig. 6a) or the expressed ARG class between H-RFI and L-RFI beef steers (Fig. 6b).

The number of total ARGs belonging to vancomycin was higher ($P = 0.044$; Fig. 7a) and aminoglycoside tended to be higher ($P = 0.067$; Fig. 7b), while MLS tended to be lower ($P = 0.053$; Fig. 7c). The abundance of bacitracin resistant genes tended to be higher ($P = 0.071$), while that of sulfonamide resistant genes ($P = 0.017$) was lower in H-RFI than L-RFI steers (Additional file 1: Table S1). The abundance of total ARG ($P = 0.071$) and MDR transcripts ($P = 0.084$) tended to be lower in H-RFI than L-RFI group (Additional file 1: Table S2). For individual gene, the abundance of *ermF* ($P = 0.004$), *InuA* ($P = 0.011$), *vatE* ($P = 0.040$), *tetX* ($P = 0.008$) was higher, and *bacA* ($P = 0.093$), *ermA* ($P = 0.100$), a MDR transporter ($P = 0.062$), *tetQ* ($P = 0.093$) tended to be higher, while that of *cat* ($P = 0.040$), *mphB* ($P = 0.027$) was lower, and class A beta-lactamase ($P = 0.079$) an ABC transporter ($P = 0.076$), *sul2* ($P = 0.092$), *vanW* ($P = 0.086$) tended to be lower in H-RFI than L-RFI group (Additional file 1: Table S1). No abundance difference in specific ARG transcript was detected between two RFI groups (Additional file 1: Table S2).

In addition, breed and feed efficiency interactively affected the abundance of chloramphenicol ($P = 0.038$) and MDR resistant genes ($P = 0.042$), as well as *fosB* ($P < 0.001$), *Isa* ($P = 0.044$), *marR* ($P = 0.007$), *mdtB* ($P < 0.001$), and *arnA* ($P = 0.045$) (Additional file 1: Table S1). No interactive effect of breed and efficiency on any expressed ARG class or individual ARG was detected (Additional file 1: Table S2).

Identification of Plasmid-associated ARGs and ARG transcripts and effect of feed and RFI on their abundance

Ten classes of ARGs were annotated as plasmid-associated by comparison against the ACLAME database, with abundances of 7.82%, 7.50%, and 7.17% for KC, AN, CH, respectively (Fig. S2a; Additional file 1: Table S3). A total of 34 of 62 (54.8%) ARGs detected were plasmid-associated (Additional file 1: Table S3). None of the abundance of plasmid-associated ARG class was affected by breed or efficiency. However, breed and efficiency interactively affected chloramphenicol resistance genes ($P = 0.016$). A total of 28, 27, and 31 plasmid-associated ARGs was detected in KC, AN, and CH, respectively, among which 22 were shared by three breeds (Fig. S2b). The abundance of *ant(9)-I* ($P = 0.006$) and *tet44* ($P = 0.035$) was higher, while that of *ermG* ($P = 0.036$), and *InuA* ($P = 0.005$) was lower in CH than KC (Fig. S2c). The abundance of *ermB* ($P = 0.008$) was higher, while that of *vatB* ($P = 0.005$) was lower in AN than CH. The abundance of *vatB* ($P = 0.003$) and *aadE* ($P = 0.038$) was higher, while that of *ermB* ($P = 0.014$) was lower, and *InuA* ($P = 0.097$) tended to be lower in KC than AN (Fig. S2c). Breed and efficiency interactively affected the abundance of *Isa* ($P = 0.038$).

Transcripts of resistance to aminoglycoside and tetracycline belonged to plasmid-associated ARGs (Additional file 1: Table S4). Transcript reads belonging to 5 of 34 detected plasmid-associated ARGs in metagenomics data were found in transcriptomic data, among which the expression of *tetW* was

observed in all samples. No effect of breed, efficiency, or their interactions on the abundance of any plasmid-associated ARG transcripts was found.

Total active bacterial population and its relationship with active resistome

The log-transformed total active bacteria copy number was lower (9.52) in KC compared with AN (11.0) and CH (10.6) ($P < 0.001$; Fig. 8a) and was not differ between H-RFI (10.4) and L-RFI (10.4) ($P = 0.963$) (Additional file 1: Table S5). No interactive effect of breed and feed efficiency was observed on total active bacterial population ($P = 0.993$).

Overall, no significant correlation was observed between total active bacteria population and the abundance (ppm) of any expressed ARG for all samples. Log-transformed total active bacteria copy number was negatively correlated with the abundance of MLS ($\rho = -0.53$, $P = 0.034$; Fig. S3a), tetracycline ($\rho = -0.54$, $P = 0.031$; Fig. S3b), and tended to be negatively correlated with that of plasmid associated tetracycline ARG transcript ($\rho = -0.48$, $P = 0.062$; Fig. S3c). For individual gene transcript, log-transformed total active bacteria copy number negatively correlated with the abundance of *mefA* ($\rho = -0.54$, $P = 0.032$; Fig. 8b), *tet40* ($\rho = -0.52$, $P = 0.037$; Fig. 8c), *tetM* ($\rho = -0.57$, $P = 0.022$; Fig. 8d), *tetW* ($\rho = -0.50$, $P = 0.050$; Fig. 8e) and an unidentified *tet* ($\rho = -0.67$, $P = 0.005$; Fig. 8f) only in KC cattle. No significant correlation was observed between log-transformed total active bacteria copy number and the abundance of any expressed ARGs in AN or CH animals, and within each RFI group.

Discussion

In this study, metagenomic analysis revealed that the abundance of ARGs in the rumen of beef steers were predominant by tetracycline (77%) and followed by MLS (17%), and aminoglycoside (4%) resistance. These findings are similar to those reported in fecal samples of beef cattle fed with antibiotics (ionophores, chlortetracycline, or tylosin), where tetracycline resistance was most prevalent (82%), followed by macrolide (14%), and aminoglycoside [32]. In addition, tetracycline, MLS, and aminoglycoside classes of resistance were also predominant in fecal samples of feedlot cattle raised without antibiotic [33], suggesting that the profiles of ARG are consistent in different locations of digestive tract of ruminants. We detected broader ARG profiles (12 classes and 62 individual ARGs) in the rumen of beef cattle not administered antimicrobials used in human medicine than previously reported by Thomas et al. [12] who studied the beef cattle supplemented with tylosin. Based on the analysis of 5 ruminal samples in cattle supplemented with monensin and tylosin, Thomas et al. [12] did not detect aminoglycoside or β -lactam resistance genes in any sample. While Auffet et al. [13] detected a wide range of genes resistant to macrolide, chloramphenicol, β -lactam, and aminoglycoside in the rumen of antimicrobial-free beef cattle under similar feeding condition to our study, however, genes resistant to vancomycin were not detected in their study. The variation could be due to difference in animal, environment and diet. In addition, bioinformatic resources/tools available for resistome analysis may

also contribute to the difference in ARG profiles in the rumen among studies. Presently, there are at least 47 bioinformatic resources/tools, but no a 'standard' pipeline has been developed specifically to characterize the resistome. In this regard, the results are heavily dependent on the analysis methods (assembly-based or read-based) or reference database [34]. In this study, the ARG-OAP (v2) pipeline was applied, which uses a custom database with a hybrid UBLAST and BLASTX algorithm, reflecting the critical need for a comprehensive database combined with lower identity matching for antimicrobial resistance gene annotation of metagenomic data [35]. However, as there is no inclusive ARG database or one specifically customized for the rumen microbiome, more efforts are needed to construct 'standardized' pipeline for to characterize the rumen resistome and resistomes in other habitats (e.g. soil, water, gastrointestinal tract).

Plasmids are mobile genetic elements found in high abundance in the bacterial populations of bovine rumen [36], which play a major role in the spread of antimicrobial resistance through horizontal gene transfer [37]. Metagenomic approaches have been used to characterize plasmid encoded ARGs in several non-biological habitats such as activated sludge [38, 39] as well as the human gut [40]. We found that *aadA* and *tetW* were the most abundant plasmid-associated ARG in the rumen of beef steers. In addition, the expression of *tetW* was highest among all plasmid-associated ARG transcripts. It has been reported that many of the tetracycline resistant genes are associated with mobile plasmids [41], among which *tetW* has been proven to be transmissible among the ruminal bacteria, *Butyrivibrio fibrosolvens*, *Selemonas ruminantium*, and *Mitsuokella multiacidus* [42]. However, the profiles and expressions of plasmid-associated ARGs have not been examined in food-producing animals including ruminants. Considering that the expression of mobile genetic elements such as integrons is a robust strategy of genetic interchange and one of the main drivers of bacterial evolution [43], we speculate that the expression of plasmid-associated ARGs has functions other than transferring antimicrobial resistance in the rumen. Recently, a wide range of bacterial hosts of plasmids in wastewater samples has been revealed by analyzing Hi-C and shotgun metagenomic data [44]. Those approaches can also be applied to investigate plasmid-associated ARG as well as their bacterial host in cattle, which may help understand the contribution of plasmids to the transmission of AMR determinants in the rumen.

Considering that the presence of a gene does not directly correlate with the activity of the gene a certain environment, direct measurements of transcripts based on metatranscriptomics may be an important complementary approach to metagenomics. Our results indicated that the expression of ARGs is also not directly linked to the presence of ARGs as previously shown in environmental microbiome [17]. We found that about only 20.96% (13/64) of ARGs were expressed, suggesting that around 80% of ARGs were not functional in the rumen of these steers at the time of sampling. Among the 13 ARGs expressed, the prevalence of *tet40*, *tetM*, *tetO*, *tetW*, *mefA* was 100%, while that of *aadA*, *tetO*, and *vatB* was 77.1%, 70.8%, and 58.3%, respectively. This suggests that these eight ARGs may constitute the 'core' active resistome in the rumen of the steers studied in our study. In particular, the average abundance (ppm) of *tetW*, *mefA*, *tetQ*, and *tet40* was 19.84, 13.61, 7.64, and 6.38, respectively, which were the predominant ARG transcripts in our study. The mechanisms of action of these resistant genes have been well characterized. Both *tet40* [45] and *mefA* [46] encode for efflux pumps which render antimicrobials

ineffective by pumping them out of the cell, while *tetQ* [47] and *tetW* [48] encode for tetracycline ribosomal protection proteins. Among these ARGs, the expression of *tet40* has been detected in *Clostridium* species in human [49] and swine [45] gut. In our previous study, we observed active *Clostridium* genus (the relative abundance averaged 0.15%) across three breeds of beef cattle [21], and we thus speculate that it may contain certain *Clostridium* species that carry *tet40* gene. To our knowledge, our study reported for the first time the presence and active ARGs simultaneously for food-producing animals *in vivo* with a large dataset. Although Sabino et al. [27] analyzed the expression of rumen ARGs, only 15 metatranscriptomic samples were used (5 dairy cattle, 5 beef cattle, and 5 sheep) and their aim was to confirm the expression of ARGs found in 435 reference genomes of ruminal bacteria and archaea *in silico*, but not to link the expression of ARGs back to the presence of those ARGs using metagenomic data. More recently, the resistome in chicken and pig gut were analyzed using both metagenomic and metatranscriptomic data, but only 6 fecal samples were used as representative of gut samples for each species [50]. In this regard, more efforts are needed to detect and validate our findings based on both metagenomic and metatranscriptomic analysis. We speculate that besides acting against antimicrobial present in the environment, the detected ARGs in the rumen may have functions in addition to antimicrobial resistance, which deserves further investigation.

It has been reported that the prevalence and abundance of ARGs in the gut of cattle is affected by diet. For example, dietary transition from milk replacer to starter led to alternation in the fecal resistome of dairy calf [7]. In addition, the diversity and abundance of total ARGs were higher in the rumen of beef cattle fed high concentrate than those fed high forage diet, with chloramphenicol and aminoglycoside resistance genes being predominant in forage- and concentrate-fed cattle, respectively [13]. A recent study also suggested that the dietary supplementation of tulathromycin, a macrolide antimicrobial drug used as metaphylaxis, significantly affected the temporal development of fecal microbiota and associated resistome in feedlot cattle [51]. To our knowledge, there is no study reporting how host genetic factors affect the active gut resistome in mammalian species. In food-producing animals such as beef cattle, understanding the 'host-resistome' association may be a prerequisite to select breeds with high feed efficiency and low risk of ARG transmission to the environment, as the gut microbiome that harbor ARGs has been proved to be largely host-driven [21, 52–54]. In this study, all beef steers were raised under the same dietary and environmental conditions, suggesting that the prevalence and expressions of ARGs were driven by host genetic factors such as breed and feed efficiency. On the contrary to the findings by Auffret et al. [13], who didn't observe breed effect on the abundance of rumen microbiota and abundance of ARGs in beef cattle, we not only observed a significant difference in both prevalence and abundance of ARGs, but also the prevalence of ARG transcripts among three breeds. Specifically, we detected less type of ARG transcripts, especially tetracycline resistant gene transcripts, in the rumen of crossbred (KC) compared with purebred (AN), which may be explained by less copy number of total active bacteria in the rumen of KC than AN animals. Besides, our previous study also indicated that the active phylum Bacteroidetes, which account for a high proportion of the microbial genomes (e.g. species belonging to *Prevotella* and *Bacteroides*) that harbor resistance genes in the rumen [27], was less abundant in KC compared with the other two breeds [21]. The significant correlation between total active bacterial

population and the abundance of ARG transcripts observed for KC cattle only further support that the expression of resistome in the rumen may be host breed specific and driven by ruminal microbiota. However, it is not clear why copy number of total active bacteria is negatively correlated with the abundance of multiple tetracycline and macrolide ARG transcripts. In this regard, the active bacterial host of those ARG transcripts deserves further investigations using the pure cultures.

It has been proved that rumen microbiome differs in beef cattle with high and low feed efficiency [21], which may explain the difference in the prevalence (e.g. ARGs belonging to vancomycin, aminoglycoside, and MLS) and abundance of several ARGs (e.g. *tetX*, *vatE*, and *InuA*) between H-RFI and L-RFI beef cattle based on metagenomic data. However, H- and L-RFI steers share a similar ARG transcript profiles, suggesting that ruminal fermentation capacity may not be a main factor driving the expression of ARGs. Our results also showed that breed × feed efficiency interactions only affect the abundance of ARGs, but not ARG transcripts. Taken together, the lack of feed efficiency and interaction effect suggest that host breed is the main drive of rumen resistome of beef steers.

Conclusions

In the current study, we not only detected a comprehensive ARG profile but also discovered 'active' resistome in the rumen of beef steers that were not administered those classes of antimicrobials used in human medicine based on both metagenomic and metatranscriptomic analysis. Our major findings include, first, not only the existence but also the expression of ARGs in the rumen are not necessarily associated with the use of antimicrobials, suggesting that the detected ARGs in the rumen may have functions in addition to antimicrobial resistance. Second, comparing with the diverse ARGs detected in the rumen, their expression level of both number and abundance of transcript is relatively low. It is plausible that there is direct relationship between the active rumen bacterial population and the active resistome. The bacterial origin, function, as well as the mechanisms of action of the active resistome needs to be verified in future studies. Third, breed exhibits a stronger effect on ARGs and their expressions compared with feed efficiency. In particular, KC has a less diversified ARG transcripts, which may be explained by the lower copy number of total active bacteria in the rumen compared with AN. Beef steers differing in RFI steers share a similar ARG transcript profiles regardless of breed, suggesting that the rumen resistome may not be a concern for future selection of beef steers with high feed efficiency. One potential limitation of the current study is that resistome was only analyzed in the rumen of monensin supplemented beef steer only, and it is unclear whether rumen resistome would have been the same if no antibiotic was administered. In this regard, comparative analysis of transcriptional profile of samples originating from cattle raised with and without antibiotics is warranted to compare the impact of the presence of antimicrobial residue on the expression of ARGs. Regardless, the findings from this study provide new insight into the active rumen resistome without the antibiotic selective pressure, which may be essential to develop strategies for limiting the spread of antimicrobial resistance from rumen to the environment.

Declarations

Availability of data and materials

Rumen metagenome and total-RNA-based metatranscriptome sequences were deposited into NCBI Sequence Read Archive (SRA) with accession number PRJNA448333.

Ethics approval and consent to participate

The present study received research ethics approval from the Livestock Care Committee of the University of Alberta (no. AUP00000882), following the guideline of the Canadian Council on Animal Care [55] (Olfert et al., 1993).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

TM and LLG designed this study. LLG and TAM provided funding. FL performed the DNA/RNA isolation, mRNA enrichment, and sequencing of library construction. TM and FL conducted bioinformatics and statistical analyses. TM, FL, TAM, RZ, and LLG were responsible for the data interpretation and manuscript writing. All authors read and approved the final manuscript.

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Figures

Figure 1

Distribution of ARGs and ARG transcripts in the rumen of 48 beef steers. a Abundance of ARGs (copies per 16S rRNA gene) in the rumen of KC, CH, and AN beef steers. b Abundance of ARGs in the rumen of H-RFI and L-RFI beef steers. c Abundance of ARG transcripts (number of ARGs sequence in one million sequence) in the rumen of AN, CH, and KC beef steers. d Abundance of ARG transcripts in the rumen of L-RFI and H-RFI beef steers. ARG, antimicrobial resistant gene; KC, Kinsella composite hybrid; AN, Angus; CH, Charolais; H-RFI, high residual feed intake; L-RFI, low residual feed intake; MLS, macrolide-lincosamide-streptogramin; MDR, multidrug.

Figure 2

Profiles of ARG classes in the rumen of 48 beef steers visualized using principle component analysis (PCA). The PCAs were plotted for (a) ARGs and (b) ARG transcripts in the rumen of KC, CH, and AN beef steers, and (c) ARGs and (d) ARG transcripts in the rumen of H-RFI and L-RFI beef steers. ARG, antimicrobial resistant gene; KC, Kinsella composite hybrid; AN, Angus; CH, Charolais; H-RFI, high residual feed intake; L-RFI, low residual feed intake.

Figure 3

Comparison of number of ARGs detected (copies per 16S rRNA gene > 0 in at least half samples for one breed) in the rumen of KC, CH, and AN beef steers. a Difference in total number of ARGs. b Difference in the number of MLS resistant genes. c Difference in the number of MDR resistant genes. d Difference in total number of vancomycin resistant genes. ARG, antimicrobial resistant gene; KC, Kinsella composite hybrid; AN, Angus; CH, Charolais; MLS, macrolide-lincosamide-streptogramin; MDR, multidrug.

Figure 4

Comparison of number of ARG transcripts in the rumen of KC, CH, and AN beef steers. a Difference in total number of ARG transcripts. b Difference in the number of tetracycline resistant transcripts. ARG, antimicrobial resistant gene; KC, Kinsella composite hybrid; AN, Angus; CH, Charolais.

Figure 5

Comparison of number of ARG transcripts in the rumen of KC, CH, and AN beef steers. a Difference in total number of ARG transcripts. b Difference in the number of tetracycline resistant transcripts. ARG, antimicrobial resistant gene; KC, Kinsella composite hybrid; AN, Angus; CH, Charolais.

Figure 6

Principle component analysis (PCA) showed no separation of the abundance of ARG classes (Fig. 6a) or the expressed ARG class between H-RFI and L-RFI beef steers (Fig. 6b).

Figure 7

Comparison of number of ARGs detected (copies per 16S rRNA gene > 0 in at least half samples for one breed) in the rumen of H-RFI and L-RFI beef steers. a Difference in total number of ARGs. b Difference in the number of aminoglycoside resistant genes. c Difference in the number of MLS resistant genes. ARG, antimicrobial resistant gene; MLS, macrolide-lincosamide-streptogramin; H-RFI, high residual feed intake; L-RFI, low residual feed intake.

Figure 8

Total active bacteria copy number and its relationship with the abundance of expressed ARGs. a Comparison of log-transformed total active bacteria copy number in the rumen of KC, AN, and CH beef steers. b Correlation between the abundance of *mefA* transcript and log-transformed total active bacteria copy number in KC beef steers. c Correlation between the abundance of *tet40* transcript and log-transformed total active bacteria copy number in KC beef steers. d Correlation between the abundance of *tetM* transcript and log-transformed total active bacteria copy number in KC beef steers. e Correlation between the abundance of *tetW* transcript and log-transformed total active bacteria copy number in KC beef steers. f Correlation between the abundance of unidentified *tet* transcript and log-transformed total active bacteria copy number in KC beef steers. KC, Kinsella composite hybrid.

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