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Insights into resistome and stress responses genes in *Bubalus bubalis* rumen through metagenomic analysis

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Abstract Buffalo rumen microbiota experience variety of diets and represents a huge reservoir of mobilome, resistome and stress responses. However, knowledge of metagenomic responses to such conditions is still rudimentary. We analyzed the metagenomes of buffalo rumen in the liquid and solid phase of the rumen biomaterial from river buffalo adapted to varying proportion of concentrate to green or dry roughages, using high-throughput sequencing to know the occurrence of antibiotics resistance genes, genetic exchange between bacterial population and environmental reservoirs. A total of 3914.94 MB data were generated from all three treatments group. The data were analysed with Metagenome rapid annotation system tools. At phyla level, Bacteroidetes were dominant in all the treatments followed by Firmicutes. Genes coding for functional responses to stress (oxidative stress and heat shock proteins) and resistome genes (resistance to antibiotics and toxic compounds, phages, transposable elements and pathogenicity islands) were prevalent in similar proportion in liquid and solid fraction of rumen metagenomes. The fluoroquinolone resistance, MDR efflux pumps and Methicillin resistance genes were broadly distributed across 11, 9, and 14 bacterial classes, respectively. Bacteria responsible for phages replication and prophages and phage packaging and rlt-like streptococcal phage genes were mostly assigned to phyla Bacteroides, Firmicutes and proteaobacteria. Also, more reads matching the sigma B genes were identified in the buffalo rumen. This study underscores the presence of diverse mechanisms of adaptation to different diet, antibiotics and other stresses in buffalo rumen, reflecting the proportional representation of major bacterial groups.

Keywords Resistome · Personal genome machine · MG-RAST · Virulence genes · Stress gene

Introduction

Livestock industry in India is subsidiary to agriculture, in the sense that animals are fed on agriculture byproduct. Feeds and fodder are in short supply and agricultural byproducts are highly lignified and poor in nutritive value. Ruminants have been bestowed upon by nature to harbor microflora and fauna which digest the fibrous roughages. In tropical countries, the buffalo are fed on lignocellulosic agricultural by-products like cereal straws and tree foliage. Ruminants digest such plant materials by microbial processes. The rumen is characterized by its high microbial population density, high diversity and complexity of interactions. Bacteria predominate in the rumen, along with a variety of anaerobic protozoa, archaea and fungi [1] and the associated occurrence of bacteriophage is also well

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documented [2]. Despite of importance of the rumen microbes to host health and productivity, knowledge about the mobilome, resistome and stress genes of bacteria remains relatively rudimentary. The feces of ruminants carries a large community of bacteria, thus it is a natural vehicle for transmission of bacteria into the environments.

Metagenomic approaches overcome the limitations of methods based on culturing or amplification [3]. Applications of metagenomics in functional selections to study antibiotic resistance and stress genes are revealing a complex network of genetic exchange between bacterial pathogens and environmental reservoirs. Using metagenomics analysis, many antibiotic resistance genes have been identified, including resistance to b-lactams [4], tetracycline [5, 6] amino glycosides [7] and bleomycin. A number of studies have also characterized antibiotic resistance genes using quantitative real-time PCR [7–10]. *Listeria monocytogenes* from Ganges water, human clinical and milk samples have been characterized by antibiotic susceptibility, serotype identification, detection of virulence genes and ERIC- and REP-PCR fingerprint analyses [11].

The role of the microbiota as reservoir of resistance genes needs to be explored in buffalo rumen. In the view of above concern, objective of the present study was to account the comparatives profiling of phylogenetic and functional potential of resistome and stress genes in *Bubalus bubalis* rumen fed different diets.

Materials and methods

Experimental design and rumen sampling

The experimental animals were maintained for feeding experiments at Livestock Research Station, Dantiwada Agricultural University, Sardarkrushinagar, Gujarat. Eight 4–5 years old healthy Mehsani breed of water buffaloes (*Bubalus bubalis*) were assigned to two basal diets groups (n = 4) based on green and dry roughages. The experimental diets were designed to have an increasing concentration of dry roughage and a decreasing concentration of the concentrate mix. The diets (dry roughage: concentrate and green roughage: concentrate) were M1D (50 % Dry roughage : 50 % concentrate), M2D (75 % Dry roughage:25 % concentrate) and M3D (100 % Dry roughage); M1G (50 % green roughage:50 % concentrate), M2G (75 % green roughage:25 % concentrate); M3G (100 % green roughage).The experimental animals received M1 diet for 6 weeks followed by M2 for 6 weeks and then M3 for subsequent 6 weeks. The animals were maintained on each diet for 6 weeks to allow for microbial adhesion and adaptation to the new diet [12]. On the last day of each experimental feeding period, rumen samples were collected 3 h post feeding using stomach tube [13]. Each rumen sample was further separated to solid and liquid fractions by squeezing through a four-layered muslin cloth. Samples were immediately placed on ice, transported to the laboratory and then stored at -80 °C prior to metagenome analyses.

DNA extraction

For isolation of DNA from liquid samples, the samples were thawed at room temperature; were then centrifuged at 5,000 rpm for 5 min. The supernatant obtained thereafter was subjected to DNA isolation using commercially available QIAmp DNA stool mini kit (Qiagen, USA). For DNA extraction from solid samples, the samples were resuspended in phosphate buffer saline and vortexed for one and half hour for dislodging the tightly adhered bacteria from the solid feed particles. The samples were then centrifuged and the supernatant was subjected to DNA isolation using the same kit which was used for liquid sample. DNA samples were measured on a Nanodrop ND-1000 spectrophotometer (Thermo Scientific) to assess DNA quantity.

Ion Torrent PGM sequencing

The shot gun sequencing on Ion Torrent PGM was performed at the Department of Animal biotechnology, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand, Gujarat, India. In brief, libraries were generated using the Ion Xpress plus fragment library kit (Life Technologies). The quality and quantity of generated libraries was assessed using the Agilent Bioanalyzer (Agilent Technologies) with Agilent High Sensitivity DNA Kit (Agilent Technologies), again quantified with Qubit florimeter (Life Technologies). Quality check passed libraries were subjected to emulsion PCR using the Ion PGM 200 Xpress Template Kit (Life Technologies). After bead enrichment, beads were loaded onto Ion 316 chips and sequenced using an Ion Torrent PGM.

Bioinformatics analysis

The data analyses were performed with Metagenome Rapid Annotation using Subsystem Technology (MG-RAST) pipelines. The reads which passed the quality filters were subjected to M5NR database (M5 non-redundant protein database, http://tools.metagenomics.anl.gov/m5nr/) for functional and diversity analysis. The M5NR is a single searchable novel non-redundant database containing protein sequences and annotations from multiple sources and associated tools. Furthermore, the functional hierarchical

Table 1 Summary of metagenomic data

Dry roughage: concentrate; green roughage: concentrate (diets)	Average read length (bp)	Data (Mb)	Total size (Mb)
50 %			
Liquid	146	549.4	1,285.4
Solid	149	736	
75 %			
Liquid	161	438	1,127.85
Solid	149	689.85	
100 %			
Liquid	180	800.69	1,501.69
Solid	170	701	
Total size		3,914.94	

classification was illustrated using SEED subsystem. The sequences were compared using the BLASTX algorithm with an expected cut off of 1×10^{-5} [8].

Analysis of similarity (ANOSIM) was used to test the null hypothesis that there is no difference between the gene content of virulence, prophage and stress response between buffalo fed 50, 75 and 100 % green and dry roughage diets. Significance was established at $P \leq 0.05$ (Tables S1, S2, and S3).

Result

Characterization of the sequencing samples

A total of 3914.94 MB data were obtained from all the samples using Ion Torrent PGM system with different reads length (145–180 bp; Table S2). The summary of metagenome data is presented in Table 1. In the present study, metagenomic sequences were used to characterize genetic diversity and functional capability of rumen microbiota of the buffalo. Analysis of community composition in rumen fluid confirmed enrichment for prokaryotic populations with high numbers.

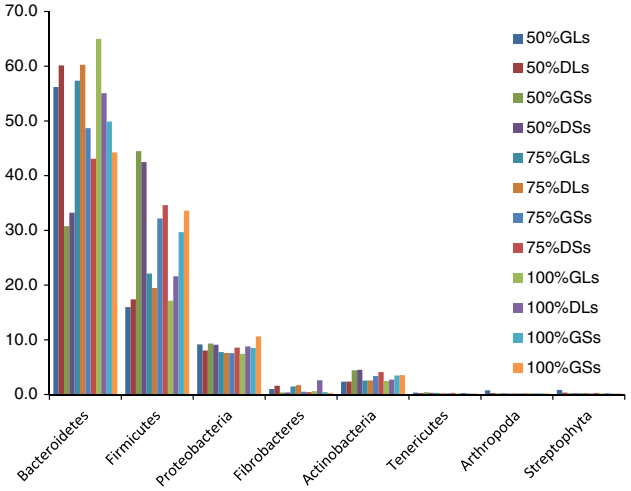

Fig. 1 Phylogenetic classification at phyla level

Using M5NR database, the domain-level breakdown of our samples revealed predominance of bacteria followed by eurkaryotes, archaea and viruses (Table 2). Majority of the eukaryotic sequences were from Fungi, Metazoa and Viridiplantae which may represent plant DNA contamination. At phyla level Bacteroidetes were dominant in all the treatments followed by Firmicutes (Fig. 1).

Functional classification of the rumen metagenomes

Analysis of the MG-RAST results indicated the presence of functionally characterized protein encoding genes (PEGs) (Supplementary File2, File 3 and File 4). Results showed that PEGs belonging to four subsystems namely, clustering-based subsystems, carbohydrates, amino acids and derivatives and protein metabolism were most abundant with similar proportion in all the metagenomes (Table S3). The subsystem database compares the homology of functional genes against the database. The functional classification by the subsystem database showed mobilome, resistome and stress genes were also abundant (Table S3).

Table 2 Phylogenetic classification at domain level

Domain	50 %				75 %				100 %			
	Green Roughage (%)		Dry Roughage (%)		Green Roughage (%)		Dry Roughage (%)		Green Roughage (%)		Dry Roughage (%)	
	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid
Bacteria	89.6	96.0	95.3	96.3	96.9	97.4	96.5	96.8	97.4	97.4	97.0	97.6
Archea	0.8	1.8	0.8	1.7	0.8	0.8	0.7	1.0	0.6	0.9	0.8	0.8
Eukaryota	9.1	1.9	3.4	1.8	1.7	1.5	2.3	2.0	1.7	1.6	1.7	1.4
Viruses	0.3	0.1	0.3	0.1	0.4	0.1	0.3	0.1	0.2	0.1	0.3	0.1
Unclassified	0.2	0.2	0.2	0.1	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2

Large numbers of genes were assigned to "clustering-based subsystems" which is often reported together in various species, however a specific function is not yet known for these genes.

Furthermore, looking to specific metabolic pathways, reads assignment with virulence, disease and defence were 2.2, 2.4 and 2.3 per cent in green liquid (GL), dry liquid (DL), green solid (GS) and dry solid (DS) in 50 % green roughage (GR) and dry roughage (DR), respectively. The GL, DL, GS and DS in 75 % GR and DR were 2.4, 2.2, 2.4 and 2.4 per cent whereas 2.2, 2.3, 2.4 and 2.4 per cent in GL, DL, GS and DS in 100 % GR and DR, respectively. Stress response genes were 1.9, 1.8, 1.9 and 1.90 percent in GL, DL, GS and DS in 50 % GR and DR, respectively. The GL, DL, GS and DS in 75 % GR and DR were 1.9, 1.8, 1.9 and 1.8 per cent whereas 1.8, 1.8, 1.9 and 1.9 percent in GL, DL, GS and DS in 100 % GR and DR, respectively (Table S3).

The subsystems-based annotations (SEED) database (MG-RAST) was utilized to gain a better understanding of metabolic potential (content of EGTs) of these microbiomes. The subsystems are annotated based on biochemical pathways, fragments of pathways, gene clusters of that function together, and any group of genes considered to be related. Figure 2 shows the SEED subsystem composition of virulence, disease and defense of buffalo rumen microbiome.The distribution of resistance to antibiotic and toxic compounds (RATC) are predominant with similar proportion in all the samples. Resistome analyses indicate that *Streptococcus agalactiae* virulome and adhesion were also abundant throughout sampling (Fig. 2).

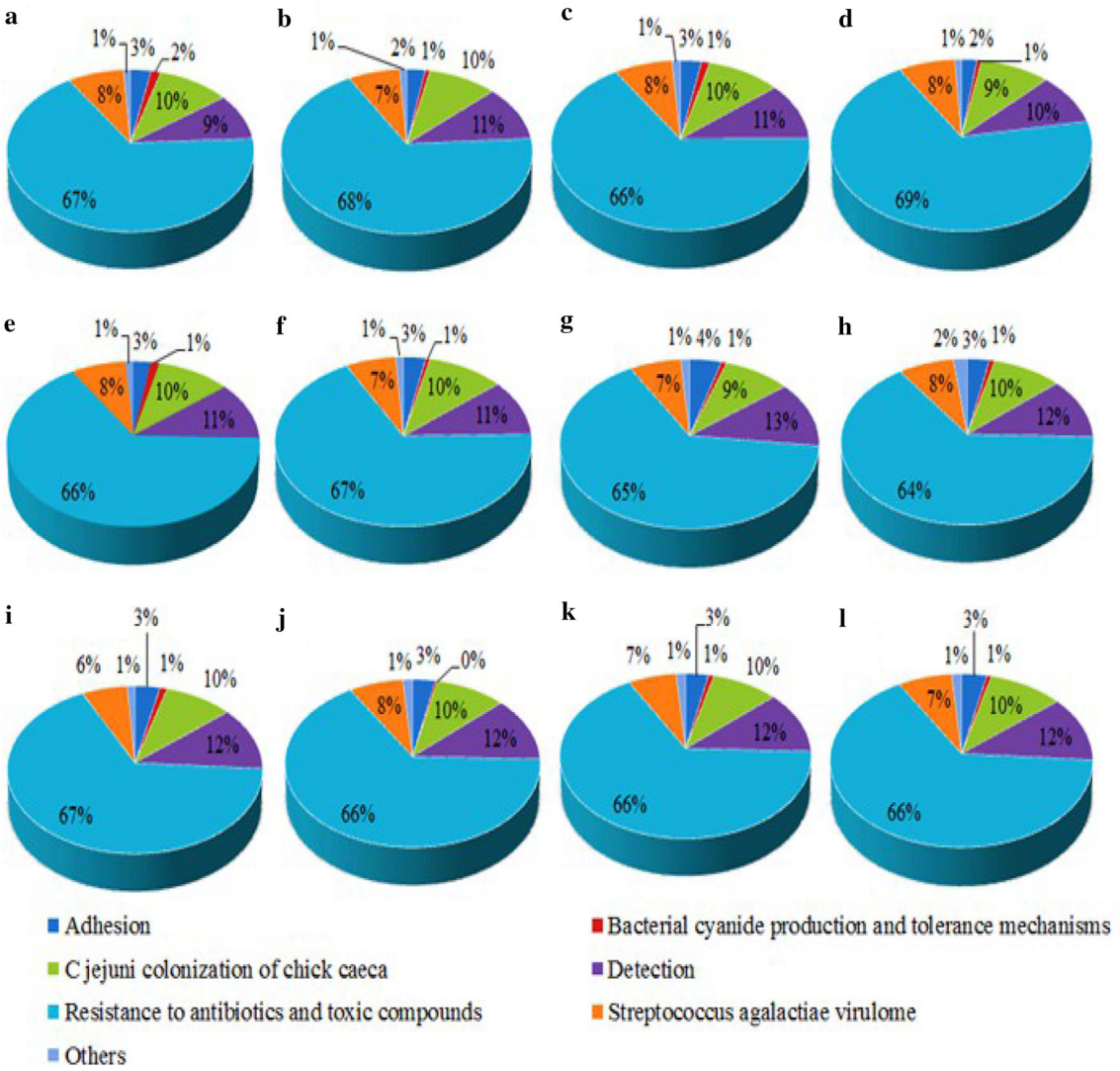

Fig. 2 SEED subsystem composition of Virulence, Disease and Defense of buffalo rumen microbiome. 50 %: **a** GL, **b** DL, **c** GS, **d** DS. 75 %: **e** GL, **f** DL, **g** GS, **h** DS. 100 %, **i** GL, **j** DL, **k** GS, **l** DS are shown. The percent of environmental gene tags (EGTs) of the SEED subsystems from the rumen microbiomes is shown. The BLASTX cut off for EGTs is 1×10^{-5}

Table 3 Abundance of resistance to antibiotics and toxic compound (RATC) genes from buffalo rumen metagenomes (%)

RATC	50 %				75 %				100 %			
	Green roughage		Dry roughage		Green roughage		Dry roughage		Green roughage		Dry roughage	
	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid
Adaptation to D-cysteine	0.16	0.33	0	0.25	0.08	0.25	0.05	0.29	0.13	0.36	0.15	0.27
Aminoglycoside adenylyltransferases	0	0.08	0	0.01	0.06	0.04	0.00	0.01	0.02	0.08	0.02	0.05
Arsenic resistance	0.04	0.30	0	0.33	0.11	0.04	0.03	0.09	0.02	0.20	0.13	0.60
Beta-lactamase	**3.00**	**2.68**	**3.38**	**1.80**	**4.53**	**3.90**	**1.94**	**2.86**	**1.56**	**5.19**	**4.33**	**4.79**
Bile hydrolysis	0.03	0.13	0	0.18	0.14	0.07	0.12	0.17	0.08	0.08	0.12	0.03
BlaR1 family regulatory sensor-transducer disambiguation	**3.88**	**9.03**	**5.06**	**7.96**	**6.69**	**8.19**	**7.32**	**8.86**	**5.72**	**9.53**	**6.75**	**9.42**
Cadmium resistance	**0.88**	**2.22**	**0.97**	**1.86**	1.48	1.65	1.09	1.78	1.47	1.99	1.07	1.28
Cobalt–zinc–cadmium resistance	**8.02**	**11.39**	**9.63**	**12.50**	**10.56**	**11.36**	**11.59**	**10.86**	**13.30**	**13.94**	**11.20**	**12.66**
Copper homeostasis	**2.14**	**6.51**	**2.75**	**5.75**	**4.26**	**5.24**	**5.32**	**6.40**	**2.92**	**6.71**	**3.96**	**6.78**
Copper homeostasis: copper tolerance	0.29	0.26	0.62	0.66	0.19	0.36	0.14	0.35	0.56	0.90	0.28	0.52
Erythromycin resistance	**2.40**	**2.52**	**3.22**	**3.19**	**1.99**	**2.31**	**2.45**	**2.70**	**2.28**	**2.97**	**2.03**	**2.70**
Fosfomycin resistance	0	0.01	0	0	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lysozyme inhibitors	0.02	0	0	0	0	0.00	0.00	0.00	0.02	0.00	0.00	0.00
Mercuric reductase	0.01	0.04	0	0.01	0.02	0.03	0.18	0.00	0.04	0.00	0.02	0.00
Mercury resistance operon	0	0.06	0	0.01	0.02	0.00	0.18	0.00	0.00	0.00	0.02	0.05
Methicillin resistance in Staphylococci	**12.81**	**9.29**	**10.86**	**9.84**	**10.22**	**10.97**	**11.41**	**10.31**	**11.47**	**12.76**	**10.32**	**10.22**
MexA–MexB–OprM multidrug efflux system	0	0	0.03	0	0	0.01	0.00	0.00	0.00	0.00	0.00	0.00
MexE-MexF-OprN Multidrug Efflux System	0.13	0.10	0.60	0.08	0.58	0.24	0.19	0.11	0.22	0.12	0.67	0.18
Multidrug resistance efflux pumps	**14.07**	**9.55**	**15.83**	**9.65**	**12.01**	**12.54**	**12.36**	**10.44**	**13.44**	**13.20**	**12.15**	**10.74**
Multidrug efflux pump in campylobacter jejuni (CmeABC operon)	**3.093**	**0.86**	**2.20**	**0.82**	**1.91**	**1.51**	**1.62**	**1.09**	**2.01**	**1.25**	**1.87**	**1.21**
Multiple antibiotic resistance MAR locus	0.03	0	0	0	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Polymyxin synthetase gene cluster in bacillus	0	0.01	0	0	0.06	0.00	0.03	0.01	0.07	0.00	0.00	0.00
Resistance to vancomycin	1.01	1.00	0.69	0.86	0.51	1.61	0.69	0.77	0.67	0.93	0.52	0.72
Resistance to chromium compounds	0	0.01	0	0	0	0.01	0.06	0.00	0.03	0.00	0.00	0.13
Resistance to fluoroquinolones	**45.81**	**42.13**	**41.97**	**42.69**	**42.23**	**37.47**	**40.90**	**40.20**	**41.35**	**41.09**	**41.91**	**35.50**
Streptococcus pneumoniae vancomycin tolerance locus	0	0	0	0	0	0.00	0.00	0.01	0.00	0.00	0.00	0.02
Streptothricin resistance	0	0	0.09	0	0	0.00	0.00	0.00	0.00	0.01	0.02	0.01
The mdtABCD multidrug resistance cluster	1.68	1.18	1.46	1.26	2.02	1.77	1.67	1.31	2.42	2.04	1.92	1.67
Zinc resistance	0.36	0.17	0.54	0.17	0.21	0.43	0.66	0.24	0.21	0.31	0.53	0.20

Bold values indicate the predominance

Among RATC, Resistance to fluoroquinolones (35.50–45.81 %), multidrug resistance efflux pumps (9.55–15.83 %) and methicillin resistance in Staphylococci (9.29–12.81 %) were predominant in all the samples (Table 3). Interestingly, methicillin resistance in staphylococci, multidrug resistance efflux pumps, multidrug efflux pump in *campylobacter jejuni* (CmeABCoperon) were more in liquid fraction of rumen metagenome fed 50 % green roughage. However, beta-lactamase was also more in liquid fraction of 50 % green roughage diet whereas also more in solid fraction of rumen fed 100 % dry roughage. Gene assignment to the BlaR1 family regulatory sensor-transducer disambiguation, cadmium resistance, cobalt-zinc-cadmium resistance, copper homeostasis and erythromycin resistance were predominant in solid fraction of rumen metagenomes (Table 3).

Metabolic potential of phages and prophages were present in all samples with low abundance. Pathogenicity and

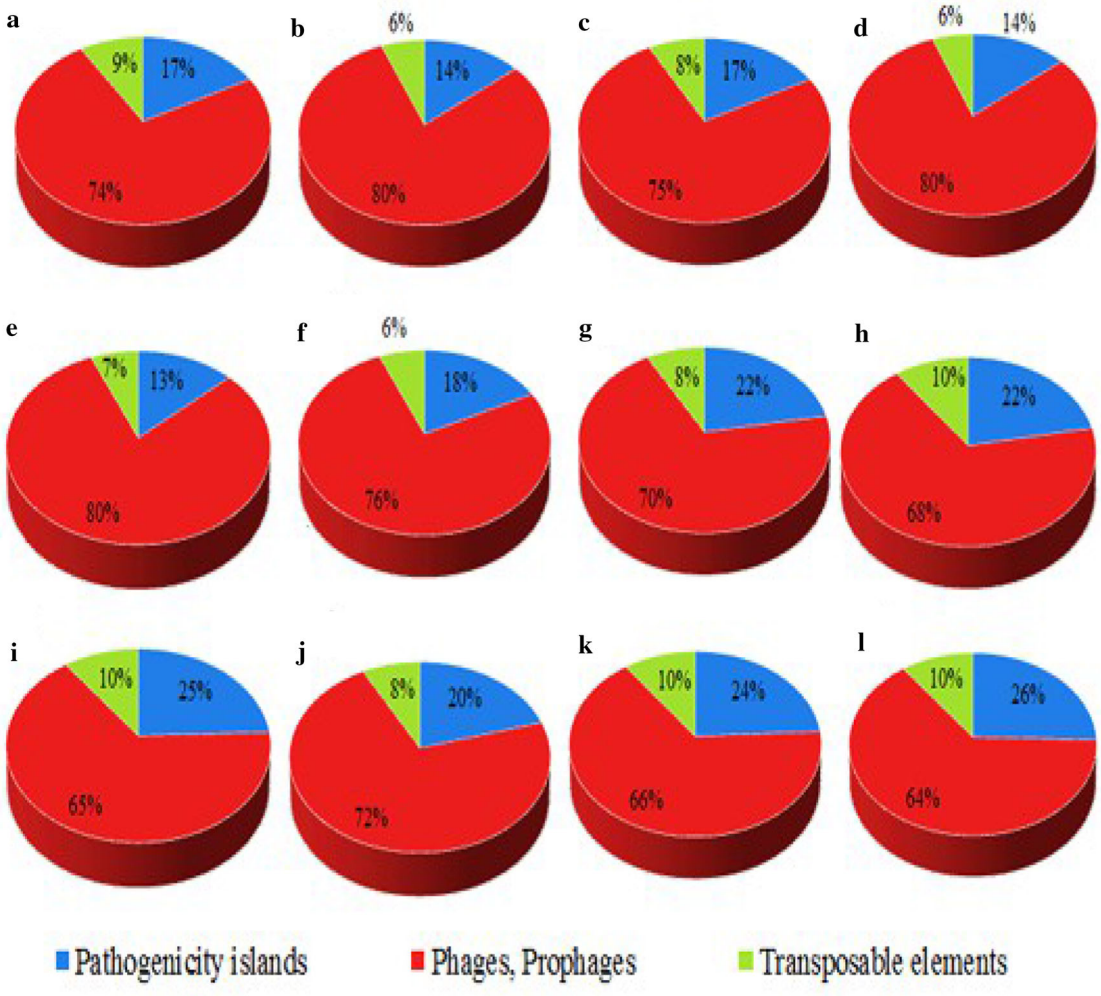

Fig. 3 SEED subsystem composition of phages of buffalo rumen microbiome. 50 %: **a** GL, **b** DL, **c** GS, **d** DS. 75 %: **e** GL, **f** DL, **g** GS, **h** DS. 100 %, **i** GL, **j** DL, **k** GS, **l** DS are shown. The percent of environmental gene tags (EGTs) of the SEED subsystems from the rumen microbiomes is shown. The BLASTX cut off for EGTs is 1×10^{-5}

transposable elements were also present with less density. However, plasmid related functions including gene transfer agent (GTA) and integrons abundances were very less (Fig. 3). Subsequently, among phages and prophages, phage replication (19.17–35.85 %), r1t-like streptococcal phages (12.72–34.86 %), phage packaging machinery (4.79–13.80 %), phage regulation of gene expression (7.35–16.54 %) and phage integration and excision (6.50–12.66 %) were predominant in the all treatments samples including liquid and solid fraction of rumen. However, interestingly appearances of genes related phage replication, phage regulation of gene expression, phage integration and excision and staphylococcal phi-Mu50B-like prophages in solid fraction were more as compared to liquid fraction in all the treatments. Although appearances of genes related r1t-like streptococcal phages, phage packaging machinery and phage tail proteins 2 in liquid fraction were more dominant as compared to solid fraction of all treatments (Table 4).

In the category of stress responses; heat shock, oxidative stress and sigmaB stress were predominant in all the samples. The detoxification, dimethylarginine metabolism, desiccation stress, cold shock, universal stress protein family, sugar-phosphate stress regulation, phage shock protein (psp) operon and Bacterial haemoglobin are very less abundant (Fig. 4; Table 5).

Among heat shock, chaperone protein DnaK (25.40–35.98 %), translation elongation factor LepA (19.71–25.01 %) and chaperone protein DnaJ (7.95–13.15 %) were predominant in the all treatments samples including liquid and solid fraction of rumen (Table S7). Furthermore assignment of genes related to chaperone protein DnaK and nucleoside 5′-triphosphatase RdgB (dHAPTP, dITP, XTP-specific) (EC 3.6.1.15) were more in liquid fraction as compared to liquid fraction in all treatments. Although appearances of genes related to Heat-inducible transcription repressor HrcA, phage packaging machine hypothetical radical SAM family enzyme, NOT coproporphyrinogen

Table 4 Abundance of phages and prophage genes in buffalo rumen metagenomes (%)

Phages and prophases	50 %				75 %				100 %			
	Green roughage		Dry roughage		Green roughage		Dry roughage		Green roughage		Dry roughage	
	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid
Phage replication	**22.92**	**28.91**	**20.44**	**31.85**	**19.17**	**28.02**	**19.34**	**36.19**	**22.84**	**33.62**	**23.08**	**35.87**
r1t-like streptococcal phages	**22.44**	**20.05**	**32.33**	**20.20**	**34.86**	**20.33**	**32.38**	**12.72**	**27.67**	**14.37**	**29.08**	**14.55**
Phage packaging machinery	**13.80**	**8.22**	**9.97**	**8.71**	**13.30**	**8.45**	**13.14**	**6.22**	**10.65**	**5.08**	**10.51**	**4.79**
Phage regulation of gene expression	**11.65**	**13.82**	**7.47**	**13.38**	**7.63**	**13.82**	**7.35**	**15.79**	**9.37**	**16.54**	**9.94**	**15.29**
Phage integration and excision	**10.02**	**12.45**	**6.74**	**11.52**	**6.75**	**11.53**	**6.50**	**13.25**	**9.57**	**12.66**	**8.76**	**12.62**
Staphylococcal phi-Mu50B-like prophages	**5.97**	**6.27**	**4.42**	**6.04**	**3.96**	**5.63**	**4.09**	**7.23**	**5.23**	**8.92**	**4.94**	**8.78**
Phage tail proteins 2	**5.10**	**2.30**	**5.32**	**1.67**	**3.90**	**3.16**	**7.12**	**1.68**	**4.43**	**1.97**	**4.09**	**1.98**
Phage capsid proteins	**2.79**	**2.68**	**4.42**	**1.97**	**3.90**	**3.84**	**3.51**	**2.01**	**3.34**	**1.89**	**4.26**	**1.80**
Phage entry and exit	**2.77**	**2.40**	**2.49**	**2.08**	**2.35**	**2.54**	**3.28**	**2.15**	**2.79**	**1.62**	**2.00**	**1.60**
Phage DNA synthesis	**1.59**	**1.69**	**1.13**	**1.78**	**1.09**	**2.04**	**1.56**	**2.06**	**1.72**	**2.61**	**1.35**	**2.11**
Listeria phi-A118-like prophages	0.43	0.16	1.02	0.24	0.59	0.17	0.78	0.20	0.75	0.16	0.72	0.05
Phage tail fiber proteins	0.31	0.30	1.81	0.03	0.84	0.10	0.11	0.00	0.38	0.14	0.35	0.03
Prophage lysogenic conversion modules	0.10	0.38	0.34	0.22	0.04	0.05	0.05	0.31	0.36	0.12	0.04	0.39
Phage baseplate proteins	0.05	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Phage introns	0.05	0.36	1.87	0.32	1.51	0.31	0.76	0.38	0.56	0.27	0.60	0.13
Phage Dual exonuclease exclusion	0.00	0.00	0.11	0.00	0.08	0.00	0.00	0.02	0.35	0.04	0.25	0.03
Phage neck proteins	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00
T7-like phage core proteins	0.00	0.00	0.00	0.00	0.02	0.00	0.02	0.00	0.00	0.00	0.00	0.00

Bold values indicate the predominance

III oxidase, oxygen-independent, MiaB family protein and possibly involved in tRNA or rRNA modification dominant in solid fraction of rumen all treatments (Table S7).

Similarly in the category of oxidative stress, Redox-dependent regulation of nucleus processes (22.47–34.97 %), Regulation of oxidative stress response (22.53–26.60 %), oxidative stress (17.30–21.80 %) and rubrerythrin (10.42–16.22 %) were predominant in the all treatments samples including liquid and solid fraction of rumen (Table S8). Although assignment of genes related to protection from reactive oxygen species was predominant in liquid fraction of rumen in all samples, gene assigned to oxidative stress and rubrerythrin were predominant in liquid fraction of all rumen samples except 100 % dry roughage diet. Surprisingly, the assignment of genes to Redox-dependent regulation of nucleus processes was higher in solid fraction of all rumen metagenome except 100 % dry roughage diets (Table S8). Phyla/class wise affiliation of resistome and stress responses genes are given in Figs. 5, 6 and 7.

Discussion

This study demonstrates that shotgun sequencing of metagenome can be used to detect the mobilome, resistome and stress responses genes from buffalo rumen metagenomes. The method for deriving rumen microbiome profiles described allows comparison of samples based on the whole population. The churning action of the rumen provides uniformity in the rumen fluid microbiome. Using SEED database, the domain-level breakdown of our samples showed bacteria, eukaryotes and viruses (Table 2). The distribution of sequences from the bacteria was in accordance with the distribution of SSU rRNA phylotypes, as reported for the canine intestinal and cattle feces microbiome studies [14, 15]. Phylogenetic potentials of buffalo rumen indicates that the Bacteroidetes were predominant, followed by Firmicutes, Proteobacteria, Actinobacteria and Fibrobacteres in all the diets (Fig. 1). Similar observation has also been reported [16] in cattle rumen and in our preliminary observation in buffalo rumen metagenome [17].

Resistome analysis

Based on SEED database functional gene categories [18], about 2.21–2.45 % metagenome sequences of all samples could be mapped to virulence genes and genes associated with RATC (Fig. 2; Table 3). Among RATC, most frequently occurring RATC functional group, fluoroquinolone

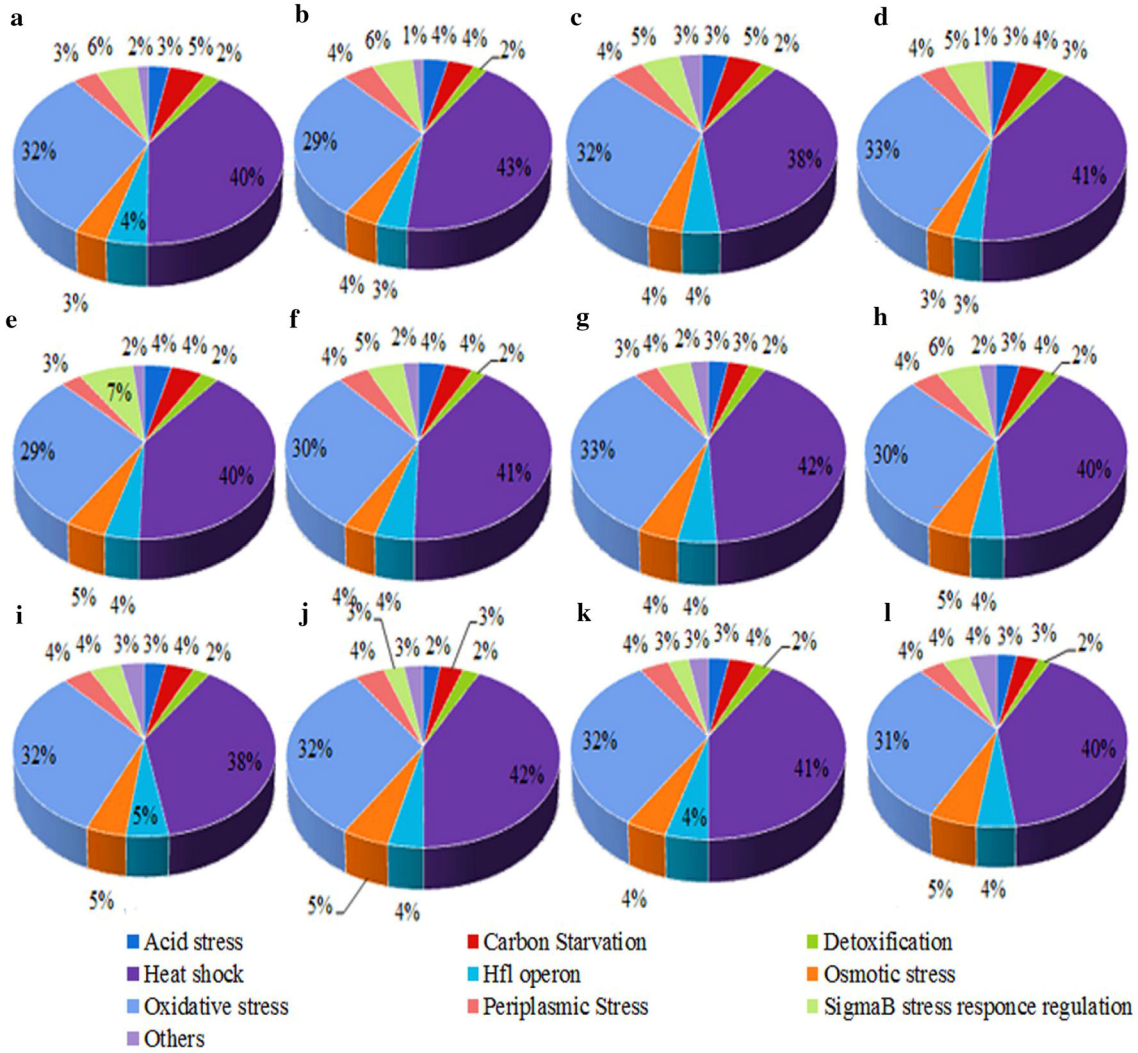

Fig. 4 SEED subsystem composition of Stress responses of buffalo rumen microbiome. 50 %: **a** GL, **b** DL, **c** GS, **d** DS. 75 %: **e** GL, **f** DL, **g** GS, **h** DS. 100 %, **i** GL, **j** DL, **k** GS, **l** DS are shown. The percent of environmental gene tags (EGTs) of the SEED subsystems from the rumen microbiomes is shown. The BLASTX cut off for EGTs is 1×10^{-5}

resistance genes (35.50–45.81 %), multidrug resistance efflux pumps (9.55–13.44 %) and Methicillin resistance in Staphylococci (9.29–12.81 %) of all virulence genes found in all buffalo rumen samples (Table 3). Similar observation has also been reported by [19] in cattle faeces microbiome, in agricultural and non-agricultural metagenomes and Cardoso et al. [20] in snail metagenome. Earlier study demonstrated that metagenomic profiles reveal biome-associated metabolic profiles, including gene assignments to the functional category of virulence. Our study extends the conclusions of Singh [13], and Dinsdale et al. [21] to include RATC, one of many subsets in the virulence category.

All metagenomes examined contained antibiotic resistance genes. Twenty-nine different RATC categories were represented in the 12 metagenomes examined (Table 3). Of these RATC categories, fluoroquinolone resistance, MDR efflux pumps, Methicillin resistance in Staphylococci, cobalt/zinc/cadmium resistance, beta-lactamase and acriflavin resistance genes were present in all metagenomes. This broad distribution across buffalo rumen samples indicates that the mechanisms of antibiotic resistance are functionally important in rumen ecosystem. This is the congruent of our preliminary results in Surti buffalo rumen [13]. Similar occurrences have been also reported in canine, fish, human feces, cattle rumen, kimchi, chicken, and termite hindgut [19]. The antibiotics resistance determinants were also identified in gypsy moth midgut [22, 23] and in swine fecal microbiome [24].

Our results indicate that the distribution of specific RATC gene categories is non-random among bacterial taxa. The fluoroquinolone resistance, MDR efflux pumps and Methicillin resistance genes were broadly distributed across 11, 9,

Table 5 Abundance of stress response genes in buffalo rumen metagenomes (%)

Stress response	50 %				75 %				100 %			
	Green roughage		Dry roughage		Green roughage		Dry roughage		Green roughage		Dry roughage	
	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid
Heat shock	**41.65**	**41.48**	**40.86**	**42.51**	**43.06**	**40.24**	**39.98**	**41.34**	**37.91**	**37.80**	**41.20**	**39.79**
Oxidative stress	**32.08**	**32.81**	**32.65**	**32.15**	**29.05**	**30.10**	**28.95**	**31.99**	**31.62**	**32.20**	**30.57**	**31.42**
Carbon starvation	**4.87**	**2.73**	**4.92**	**3.00**	**3.64**	**3.62**	**4.29**	**3.55**	**4.59**	**3.93**	**3.69**	**3.03**
Hfl operon	**4.41**	**3.86**	**2.31**	**3.77**	**3.39**	**3.67**	**3.80**	**4.46**	**4.03**	**4.67**	**4.32**	**4.47**
Osmotic stress	**3.65**	**4.18**	**2.67**	**4.95**	**3.98**	**4.90**	**4.50**	**4.33**	**3.70**	**4.30**	**3.63**	**5.41**
Periplasmic stress	**3.52**	**3.22**	**4.55**	**3.98**	**4.51**	**3.93**	**3.04**	**3.93**	**4.45**	**4.07**	**4.07**	**3.50**
Acid stress	**2.58**	**2.58**	**3.12**	**2.28**	**3.38**	**3.23**	**3.82**	**2.77**	**3.55**	**3.07**	**3.61**	**2.90**
Dessication stress	0.24	0.13	0.26	0.10	0.08	0.11	0.06	0.21	0.12	0.08	0.01	0.06
Bacterial hemoglobins	0.00	0.19	0.11	0.31	0.00	0.00	0.23	0.00	0.29	0.06	0.14	0.56
Cold shock	0.09	0.24	0.00	0.14	0.12	0.11	0.23	0.14	0.05	0.12	0.08	0.28
Commensurate regulon activation	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03
Detoxification	**1.78**	**2.31**	**2.67**	**2.20**	**1.91**	**2.08**	**2.45**	**2.38**	**2.02**	**2.29**	**1.92**	**1.72**
Dimethylarginine metabolism	0.86	1.74	0.44	1.74	1.29	2.15	1.06	2.18	2.44	2.88	1.74	2.94
Flavohaemoglobin	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.07	0.00	0.08
Phage shock protein (psp) operon	0.02	0.15	0.19	0.09	0.00	0.00	0.04	0.09	0.12	0.05	0.10	0.07
SigmaB stress responce regulation	**4.13**	**4.36**	**5.24**	**2.78**	**5.57**	**5.87**	**7.50**	**2.61**	**5.09**	**4.41**	**4.91**	**3.68**
Sugar-phosphate stress regulation	0.04	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00
Universal stress protein family	0.08	0.01	0.00	0.00	0.03	0.00	0.00	0.02	0.00	0.00	0.00	0.05

Bold values indicate the predominance

and 14 bacterial classes, respectively (Fig. 5). Bacteria responsible for fluoroquinolone resistance, MDR efflux pumps and Methicillin resistance genes were mostly assigned to phyla Bacteroides, Firmicutes and proteaobacteria in all 12 metagenomes (Fig. 5). Similar occurrences have been also reported by Durso et al. [19] and they concluded that the MDR efflux genes were most frequently assigned to Clostridia in the animal agriculture samples and Gammaproteobacteria in the coastal marine samples.

Our results support those of Reyes et al. [25] describing a global pool of antibiotic resistance genes. Studies showing that the occurrence of antibiotic resistance is an ancient phenomena [26], as it can be found in a variety of human-impacted and pristine habitats [27, 28]. Our study further supports the notion that the presence of antibiotic resistant genes is a normal and natural phenomenon. Hence, while emphasizing the impacts of veterinary use of antibiotics on human health, baseline studies and control samples are required to assess natural prevalence of antibiotic resistant bacteria and/or antibiotic resistance genes for comparison.

Functional analysis of phages reveals fitness genes

Seed subsystem composition of phages of buffalo rumen microbiome indicates the predominance of pathogenicity islands (Fig. 3). Phages replication and Prophages and phage packaging and rlt streptococcal phage genes were broadly distributed across15, 11 and 12 bacterial classes in all 12 metagenomes (Fig. 6). Our results show that appearance of phage encoding genes in buffalo rumen, reflecting the induction of prophages in rumen bacteria. Genes related to integrases and pathogenicity islands have also been detected by [29] in phages. Allen et al. [24] have detected resistance gene in the swine viruses and frequency of antibiotic resistance genes in an *Escherichia coli* genome. Resistance genes were identified slightly more frequently in human fecal viromes [30]. Phages have been shown to play an important role in ecosystem dynamics [31].

Genes involved in stresses

Genes implicated in adaptation to stress responses [29] were present in all twelve metagenomes, as shown in Tables S3 and S7. These included the genes encoding Chaperone protein (DnaK), Chaperone protein (DnaJ) and nucleoside 5′-triphoaphate RdgB were predominant and play importance in adaptations to psychrophilic lifestyles [32]. Chaperone protein (DnaK) genes were broadly distributed across 10 bacterial classes (Fig. 7).Conversely, the buffalo rumen had a higher representation of the alternative

a

Phylum	Class	50% Roughage				75% Roughage				100% Roughage			
		GL	DL	GS	DS	GL	DL	GS	DS	GL	DL	GS	DS
Bacteroidetes	Bacteroidia	8.74	30.21	20.81	16.00	30.61	21.85	17.17	18.31	11.86	19.35	19.20	21.13
Firmicutes	Clostridia	28.42	17.19	18.50	30.86	32.65	19.33	16.16	18.78	10.67	18.43	18.00	16.43
Actinobacteria	Actinobacteridae	9.84	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Flavobacteriia	14.75	13.54	34.10	14.86	19.73	33.61	46.46	28.64	20.55	29.49	16.80	19.25
Cyanobacteria	Chroococcales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Erysipelotrichi	13.66	0.00	12.14	24.00	0.00	0.00	0.00	0.00	0.00	7.83	12.80	0.00
Firmicutes	Bacilli	10.38	16.67	0.00	0.00	17.01	25.21	20.20	13.62	13.83	15.67	20.00	15.96
Dictyoglomi	Dictyoglomales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Deltaproteobacteria	0.00	0.00	14.45	14.29	0.00	0.00	0.00	0.00	9.09	0.00	0.00	0.00
Actinobacteria	Rubrobacteridae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Cytophagia	5.46	0.00	0.00	0.00	0.00	0.00	0.00	0.00	14.23	9.22	13.20	0.00
Chlorobi	Chlorobia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Negativicutes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	20.66	0.00	0.00	0.00	12.21
Bacteroidetes	Sphingobacteriia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	15.02
Fusobacteria	Fusobacteriales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Gammaproteobacteria	8.74	22.40	0.00	0.00	0.00	0.00	0.00	0.00	19.76	0.00	0.00	0.00
	Total %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

b

Phylum	Class	50% Roughage				75% Roughage				100% Roughage			
		GL	DL	GS	DS	GL	DL	GS	DS	GL	DL	GS	DS
Bacteroidetes	Bacteroidia	52.38	56.10	38.60	46.67	64.29	13.89	16.04	17.98	30.00	14.42	34.43	40.00
Firmicutes	Clostridia	47.62	43.90	61.40	53.33	35.71	29.86	24.53	0.00	21.00	25.96	32.79	60.00
Actinobacteria	Actinobacteridae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Flavobacteriia	0.00	0.00	0.00	0.00	0.00	29.86	0.00	0.00	0.00	0.00	0.00	0.00
Cyanobacteria	Chroococcales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	42.70	0.00	0.00	0.00	0.00
Firmicutes	Erysipelotrichi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	20.22	35.00	27.88	0.00	0.00
Firmicutes	Bacilli	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dictyoglomi	Dictyoglomales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Deltaproteobacteria	0.00	0.00	0.00	0.00	0.00	9.72	33.02	19.10	0.00	31.73	0.00	0.00
Actinobacteria	Rubrobacteridae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Cytophagia	0.00	0.00	0.00	0.00	0.00	16.67	26.42	0.00	14.00	0.00	0.00	0.00
Chlorobi	Chlorobia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	32.79	0.00
Firmicutes	Negativicutes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Sphingobacteriia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fusobacteria	Fusobacteriales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Total %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

c

Phylum	Class	50% Roughage				75% Roughage				100% Roughage			
		GL	DL	GS	DS	GL	DL	GS	DS	GL	DL	GS	DS
Bacteroidetes	Bacteroidia	21.94	43.51	18.62	28.36	33.33	12.21	27.27	20.50	18.92	52.24	52.73	40.00
Firmicutes	Clostridia	14.84	0.00	9.66	14.18	33.33	29.01	16.53	14.91	18.92	0.00	47.27	26.67
Actinobacteria	Actinobacteridae	0.00	0.00	0.00	7.46	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Flavobacteriia	23.23	26.62	16.55	16.42	33.33	40.46	27.27	6.21	0.00	0.00	0.00	0.00
Cyanobacteria	Chroococcales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Erysipelotrichi	23.87	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	47.76	0.00	33.33
Firmicutes	Bacilli	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	41.44	0.00	0.00	0.00
Dictyoglomi	Dictyoglomales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	20.72	0.00	0.00	0.00
Proteobacteria	Deltaproteobacteria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	14.91	0.00	0.00	0.00	0.00
Actinobacteria	Rubrobacteridae	0.00	0.00	19.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Cytophagia	0.00	0.00	15.17	33.58	0.00	18.32	0.00	12.42	0.00	0.00	0.00	0.00
Chlorobi	Chlorobia	16.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Negativicutes	0.00	0.00	20.69	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Sphingobacteriia	0.00	29.87	0.00	0.00	0.00	0.00	28.93	9.94	0.00	0.00	0.00	0.00
Fusobacteria	Fusobacteriales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	21.12	0.00	0.00	0.00	0.00
	Total %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Fig. 5 Bacteria responsible for **a** fluoroquinolones genes, **b** multidrug resistance efflux pumps genes, and **c** methicillin resistance in Staphylococci genes in 12 metagenomes. Percent of genes within each metagenome that are assigned to the listed taxa

sigma factor (sigma B) gene, which is a general stress regulon that induces many genes in response to variety of stresses, including heat, acid, salt, and starvation [33]. Matches assigned to the genes of more-constitutive proteins associated with cold adaptation chaperones DnaK and DnaJ, were also abundant in the buffalo rumen microbial

a

Phylum	Class	50% Roughage				75% Roughage				100% Roughage			
		GL	DL	GS	DS	GL	DL	GS	DS	GL	DL	GS	DS
Bacteroidetes	Bacteroidia	23.93	17.95	19.08	19.23	38.66	24.86	19.64	30.73	15.48	21.36	15.09	30.00
Firmicutes	Clostridia	16.56	9.83	9.16	20.09	25.21	12.72	19.64	21.79	16.41	21.36	16.38	18.24
Actinobacteria	Actinobacteridae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Flavobacteriia	14.72	10.26	4.96	11.11	14.29	16.18	14.88	27.37	8.05	0.00	13.36	0.00
Cyanobacteria	Chroococcales	0.00	0.00	0.00	8.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Erysipelotrichi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Bacilli	0.00	14.53	20.99	20.51	0.00	9.83	24.40	0.00	16.41	8.18	19.83	26.47
Dictyoglomi	Dictyoglomales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Deltaproteobacteria	30.06	11.97	7.25	0.00	0.00	12.72	0.00	0.00	0.00	13.18	0.00	0.00
Actinobacteria	Rubrobacteridae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Cytophagia	0.00	0.00	11.83	20.51	0.00	23.70	0.00	0.00	6.19	15.91	18.53	14.71
Chlorobi	Chlorobia	0.00	0.00	17.56	0.00	0.00	0.00	0.00	0.00	12.38	10.91	0.00	0.00
Firmicutes	Negativicutes	0.00	0.00	0.00	0.00	0.00	0.00	21.43	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Sphingobacteriia	0.00	23.50	0.00	0.00	21.85	0.00	0.00	20.11	14.55	9.09	16.81	10.59
Fusobacteria	Fusobacteriales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Gammaproteobacteria	14.72	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.53	0.00	0.00	0.00
Tenericutes	Mollicutes	0.00	11.97	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Spirochaetes	Spirochaetales	0.00	0.00	9.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Total %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

b

Phylum	Class	50% Roughage				75% Roughage				100% Roughage			
		GL	DL	GS	DS	GL	DL	GS	DS	GL	DL	GS	DS
Bacteroidetes	Bacteroidia	23.50	15.22	16.17	16.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Clostridia	14.21	7.97	8.68	15.04	19.50	20.23	25.00	17.65	32.20	26.17	23.31	21.50
Actinobacteria	Actinobacteridae	0.00	0.00	0.00	0.00	21.00	21.39	0.00	0.00	14.12	12.75	18.05	0.00
Bacteroidetes	Flavobacteriia	14.75	11.23	14.37	10.16	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cyanobacteria	Chroococcales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Erysipelotrichi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Bacilli	0.00	12.32	15.87	13.82	22.00	13.87	25.86	16.91	16.95	18.79	15.79	21.50
Dictyoglomi	Dictyoglomales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Deltaproteobacteria	5.46	0.00	8.68	17.48	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Actinobacteria	Rubrobacteridae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Cytophagia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chlorobi	Chlorobia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Negativicutes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Sphingobacteriia	0.00	16.30	5.69	5.69	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Fusobacteria	Fusobacteriales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Gammaproteobacteria	13.11	15.22	13.17	0.00	11.00	13.29	0.00	16.91	0.00	0.00	0.00	0.00
Tenericutes	Mollicutes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Spirochaetes	Spirochaetales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
dsDNA viruses	no RNA stage	13.66	9.78	8.38	10.16	14.50	16.76	20.69	19.85	16.38	23.49	23.31	23.36
Chloroflexi	Dehalococcoidetes	15.30	11.96	8.98	10.98	12.00	14.45	28.45	28.68	20.34	18.79	19.55	33.64
	Total %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

c

Phylum	Class	50% Roughage				75% Roughage				100% Roughage			
		GL	DL	GS	DS	GL	DL	GS	DS	GL	DL	GS	DS
Bacteroidetes	Bacteroidia	21.83	18.82	13.79	22.75	24.24	18.38	23.81	33.55	31.53	32.93	27.17	29.17
Firmicutes	Clostridia	16.20	9.68	14.37	13.23	21.21	12.43	19.05	9.68	17.12	17.07	20.65	31.25
Actinobacteria	Actinobacteridae	0.00	0.00	0.00	13.23	0.00	0.00	8.57	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Flavobacteriia	0.00	0.00	0.00	0.00	31.82	29.73	10.00	0.00	27.03	16.46	9.24	12.50
Cyanobacteria	Chroococcales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Erysipelotrichi	14.08	9.68	0.00	21.16	0.00	0.00	0.00	0.00	24.32	14.02	10.87	0.00
Firmicutes	Bacilli	18.31	10.75	18.97	9.52	12.88	11.35	16.19	21.94	0.00	0.00	0.00	0.00
Dictyoglomi	Dictyoglomales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Deltaproteobacteria	0.00	0.00	0.00	0.00	0.00	17.30	11.90	21.29	0.00	0.00	0.00	0.00
Actinobacteria	Rubrobacteridae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Cytophagia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chlorobi	Chlorobia	0.00	6.45	11.49	0.00	9.85	10.81	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Negativicutes	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Sphingobacteriia	13.38	24.19	17.24	8.99	0.00	0.00	0.00	0.00	0.00	0.00	15.76	27.08
Fusobacteria	Fusobacteriales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Gammaproteobacteria	0.00	7.53	12.64	0.00	0.00	0.00	10.48	13.55	0.00	19.51	16.30	0.00
Tenericutes	Mollicutes	16.20	12.90	11.49	11.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Spirochaetes	Spirochaetales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Total %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Fig. 6 Bacteria responsible for **a** phage replication of phage and prophages genes, **b** Phage packaging-phage's and Prophages genes and **c** r1t-like streptococcal phages genes in 12 metagenomes. Percent of genes within each metagenome that are assigned to the listed taxa

communities. These genes are known to be induced in bacteria upon exposure to cold temperatures [34].

The rumen metagenomic study revealed the Resistome and Stress response genes present in Indian buffalo (*Bubulas bubalis*) rumen. The genes coding for functional responses to stress and resistome genes, phages, transposable elements and pathogenicity islands) were prevalent in similar proportion in both fraction of ruminal fluid

		50% Roughage				75% Roughage				100% Roughage			
Phylum	Class	GL	DL	GS	DS	GL	DL	GS	DS	GL	DL	GS	DS
Bacteroidetes	Bacteroidia	21.05	33.33	36.11	35.56	21.66	48.91	28.32	22.39	17.82	22.88	42.86	28.21
Firmicutes	Clostridia	40.35	34.67	46.30	34.81	18.47	18.48	33.63	29.85	16.09	22.88	11.43	18.59
Actinobacteria	Actinobacteridae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	20.00	0.00
Bacteroidetes	Flavobacteriia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	11.49	23.53	0.00	33.97
Cyanobacteria	Chroococcales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Erysipelotrichi	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Bacilli	14.04	32.00	0.00	0.00	0.00	0.00	0.00	0.00	16.67	0.00	0.00	0.00
Dictyoglomi	Dictyoglomales	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Proteobacteria	Deltaproteobacteria	0.00	0.00	0.00	0.00	17.20	0.00	0.00	15.67	16.09	0.00	0.00	0.00
Actinobacteria	Rubrobacteridae	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Cytophagia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chlorobi	Chlorobia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Firmicutes	Negativicutes	0.00	0.00	17.59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteroidetes	Sphingobacteriia	0.00	0.00	0.00	0.00	0.00	0.00	38.05	0.00	0.00	0.00	0.00	0.00
Fusobacteria	Fusobacteriales	0.00	0.00	0.00	29.63	17.20	32.61	0.00	0.00	21.84	30.72	0.00	19.23
Proteobacteria	Gammaproteobacteria	24.56	0.00	0.00	0.00	25.48	0.00	0.00	32.09	0.00	0.00	25.71	0.00
	Total %	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Fig. 7 Bacteria responsible for dnaK genes in 12 metagenomes. Percent of dnaK genes within each metagenome that are assigned to the listed taxa

biomaterials. Metagenomic RATC gene data can be used to link antibiotic resistance information with bacterial community composition in agriculturally impacted environments. The present study provides a baseline for understanding the complexity of the microbial ecology of the buffalo rumen with special reference to resistome, mobilome and stress responses.

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