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Metagenomic analysis of buffalo rumen microbiome: Effect of roughage diet on Dormancy and Sporulation genes

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ABSTRACT

Buffalo rumen microbiome experiences a variety of diet stress and represents reservoir of Dormancy and Sporulation genes. However, the information on genomic responses to such conditions is very limited. The Ion Torrent PGM next generation sequencing technology was used to characterize general microbial diversity and the repertoire of microbial genes present, including genes associated with Dormancy and Sporulation in Mehsani buffalo rumen metagenome. The research findings revealed the abundance of bacteria at the domain level and presence of Dormancy and Sporulation genes which were predominantly associated with the Clostridia and Bacilli taxa belonging to the phyla Firmicutes. Genes associated with Sporulation cluster and Sporulation orphans were increased from 50% to 100% roughage treatment, thereby promoting sporulation all along the treatments. The spore germination is observed to be the highest in the 75% roughage treatment both in the liquid and solid rumen fraction samples with respect to the decrease in the values of the genes associated with spore core dehydration, thereby facilitating spore core hydration which is necessary for spore germination.

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1. Introduction

The rumen is characterized by 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 (Hespell et al., 1997) and the associated occurrence of bacteriophage. The rumen can be assumed as a kind of anaerobic fermentation tank, in which rumen microorganisms interact with each other (Moto, 2008). Microbes survive in the rumen under different constraints which may be either natural or feed associated, as some of the feeds contain a significant amount of anti-nutritional factors, which sometimes limit the growth of some of these natural microbial inhabitants (Sahu et al., 2004). Many workers have reported influences of multiple factors on the composition of rumen microbiota and thus affecting the population of certain bacterial groups (Fonty et al., 1987; Romero-Pérez et al., 2011).

Bacteria have the ability to adapt to different growth conditions and to survive in various environments. They also have the capacity to enter into dormant states. Some bacteria form spores when exposed to stresses such as starvation and oxygen deprivation (Bhupender et al., 2010). Endospores are dormant, non-reproductive and enzymatically inert forms of bacterial vegetative cells (Cano and M.K.B., 2012). Endospore-producing bacteria are also conventionally termed as “sporulating bacteria” (Cano and M.K.B., 2012).

The spores serve to protect the bacterium from harmful environmental conditions by reducing into a desiccated, cryptobiotic and highly defensive state, which provides resistance to many environmental conditions that would otherwise harm and kill the vegetative form of the bacterium. These environmental conditions include extreme temperatures, radiation, extreme pH levels, extreme pressures and harmful chemical agents (Cano and M.K.B., 2012). Breaking of the dormant state of bacterial spores is the initiating event in germination. Glucose, certain amino acids, nucleosides, and even salts are among the many agents which induce initiation, however the mechanism is not understood (Woese et al., 1968). Uncultured bacteria are predicted to be a significant reservoir of novel small-molecule biosynthetic machinery (Brady et al., 1998; Rondon et al., 2000). Functional metagenomics is one of the approaches by which one can access the biosynthetic potential contained within the genomes of uncultured bacteria (Brady et al., 1998). Despite of importance of the rumen microbial population to host health and productivity, knowledge about Dormancy and Sporulation and Cell wall and Capsule genes of bacteria remains relatively rudimentary. Massive parallel sequencing (MPS) of metagenome DNA without targeted amplification, termed metagenomics, avoids these issues while enabling a substantial increase in the volume of data produced. In addition, the cost of MPS is falling rapidly. Untargeted MPS involves extracting whole DNA or RNA from the community or tissue of interest (Hess et al., 2011).

The metagenomics RAST (MG-RAST) server is a fully automated open source system for processing metagenome sequence. A public accessibility of this system has been provided for all researchers to analyse their metagenome. MG-RAST is available over the web to all researchers, and access is not limited to specific groups or data types. The MG-RAST server is an open source system based on the SEED framework for comparative genomics (Tyson et al., 2004; Huse, 2007). This approach highlights subsystems that are unique to a metagenome, a comparative genome, or the subsystems common to both. With the identification of Dormancy and Sporulation associated bacterial genes in the buffalo rumen, the role of the microbiota as a reservoir of dormant state in bacterial spores' genes needs to be explored. The objective of the present study was to make a comparative analysis at the functional level with respect to the SEED subsystem Dormancy and Sporulation, Cell wall and Capsule and Fatty acids, lipids and Isoprenoids of *Bubalus bubalis* rumen metagenome sample.

2. Materials and methods

2.1. Sample collection

Eight healthy Mehsani buffaloes were reared at Livestock Research Station, Sardar Krushinagar Agriculture University. Out of eight, four animals were fed on 50% Dry roughage and 50% concentrate, followed by 75% Dry roughage and 25% concentrate and then 100% dry roughage, while another four animals were fed on 50% green roughage and 50% concentrate, followed by 75% green roughage and 25% concentrate and then 100% green roughage over a period of one month. Thereafter samples were collected

from the rumen of the buffalo, in the last week of treatment at 2 and ½ h after morning feeding using a flexible stomach tube. Approximately 500 ml of the rumen fluid collected was filtered through muslin cloth and the filtrate was aliquoted into the 2 ml cryo-vials. The solid fraction was also aliquoted into 2 ml cryo-vials. These vials were then immediately frozen into liquid nitrogen.

2.2. DNA extraction

DNA extraction was carried out from 200 ml of the green and dry liquid rumen samples as well as 200 mg of the green and dry solid rumen samples using the commercially available QIAamp DNA stool mini kit (Qiagen, USA). Metagenome was quantified by using the Nanodrop Spectrophotometer.

2.3. Shotgun sequencing

The Ion Xpress™ Fragment Library Kit (Life Technologies, Carlsbad, CA) was used to construct a library for shotgun sequencing on the Ion Personal Genome Machine (PGM, Ion Torrent/Life Technologies). The DNA was subjected to enzymatic fragmentation and library was constructed using the Ion Fragment Library Kit protocol. Adaptors were ligated to the repaired fragment ends followed by size selection. The library was PCR amplified using forward and reverse primers. The quality and the quantity of each of the libraries were assessed with the 2100 Bioanalyzer (DNA High Sensitivity Chip, Agilent Technologies, Sunnyvale, CA). Templates were then prepared and enriched on the Ion Sphere Particles™ (ISPs) using the Ion Xpress™ Template Kit (Life Technologies) and subjected to sequencing using the Ion Express Template 200 kit (Life Technologies, USA). Signal processing and base calling were performed with Torrent Analysis Suite version 3.4.1.

2.4. Data analysis

The sequence data were uploaded onto the publically available server MG RAST (Meta Genome Rapid Annotation using Subsystem Technology, v3.3). The taxonomic domain groups were assigned using MG RAST against M5NR database, which is an integration of many sequence databases into a single and searchable database. A single similarity search at this server will allow retrieving similarities to several databases, including NCBI-nr, KEGG, and SEED. Global gene expressions were annotated with the SEED Subsystems in MG RAST with the datasets having a cut-off of e-value $<1e^{-5}$, % identity cut-off of 60% and minimum alignment cut-off of 15 bp to get the metabolic pathway information.

2.5. GO Slim analysis

The Gene Ontology analysis was performed using GO Slim. GO Slims are cut down versions of gene ontologies containing a subset of the terms in the whole GO. The reads corresponding to the 50%, 75% and 100% green and dry roughage treatments were analysed with the Interpro database and the hit values generated were used in the GO Slim analysis which gave a broad overview of the ontology content without the detail of specific fine grained terms.

2.6. Statistical analysis

Standard errors for the datasets were calculated using the SPSS Statistics software v17 and One-way ANOSIM through the PAST tool was performed to check for the statistical significance of differences between results obtained for the different feed treatments. The ANOSIM test statistic, *R*, is a measure of the magnitude of dissimilarity within where *R* values near 0 that indicate dissimilarities between sample points within one group are equivalent to the dissimilarities found between different groups. Statistical significances in the form of *P* values were calculated for each pair-wise *R* value (Clarke, 1993).

Table 1
The output of the sequencing runs of the rumen samples on Ion Torrent PGM (In the table the output for the Liquid and Solid samples for each treatment is in terms of the average of four samples each for liquid and solid groups).

Roughage treatment		Data in (Mbp)	Mean read length (bp)	Post QC sequence count	Total number of reads
50% (M1)	Liquid	549.4	146	18,04,783	18,91,804
	Solid	736.0	149	26,30,228	29,13,490
75% (M2)	Liquid	438.0	161	18,77,607	21,25,995
	Solid	689.9	149	31,99,080	32,32,711
100% (M3)	Liquid	800.7	180	32,27,832	35,57,735
	Solid	701.0	170	36,34,016	40,16,804

3. Result

Next generation sequencing has lead to the characterization and identification of the functional capacity of the rumen microbial communities. The summary of metagenome data is presented in Table 1. In the present study, metagenomic sequences were used to characterize genetic diversity and functional

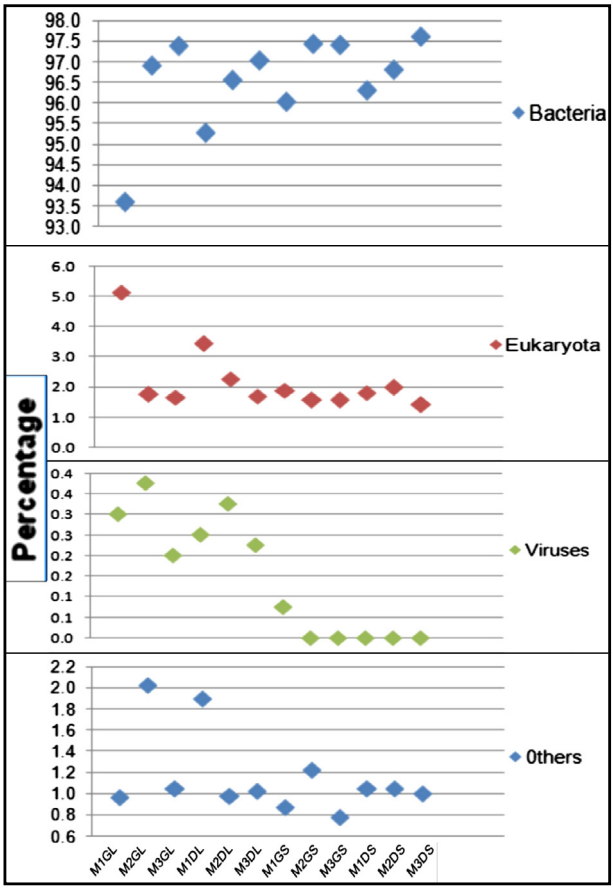

Fig. 1. The taxonomic domain information of the DNA datasets. Total DNA sequences were assigned to bacteria, eukaryota, viruses, unclassified and other sequences.

capability of the rumen microbiota of the buffalo. Analysis of community composition in rumen fluid confirmed enrichment for prokaryotic populations with high numbers.

3.1. Taxonomic assignment

After being filtered by MG-RAST based on length and number of ambiguous bases, the datasets were used for the taxonomic analysis. Using M5NR database, the domain-level breakdown of our samples was predominated by bacteria followed by eukaryotes, archaea and viruses (Fig. 1), accounting for a minimum of 89.60% in M1 Green liquid to a maximum of 97.6% in M3 Dry solid, while eukaryote comprised approximately 1.40% to 9.10% of total sequences in the DNA from the samples. Viruses accounted for 0.2% to 0.3% in the DNA sequences from most of the samples.

3.2. Predicted gene functions

Analysis of the MGRAST results indicated the presence of functionally characterized protein encoding genes (PEGs) (Tables 2 and 3). The highest proportion of gene fragments assigned to known functions was associated with clustering based subsystems ranging from 15.46% to 16.63%, followed by genes associated with carbohydrate metabolism (11.22% to 12.61%) and then protein metabolism (11.10% to 12.16%). The genes associated with Dormancy and sporulation, Cell wall and Capsule and Fatty acids, lipids and Isoprenoids ranged between 0.33% and 0.43%, 4.23% and 5.21% and 1.74% and 2.03% respectively (Fig. 2A and B). One-way ANOSIM analysis showed significant difference in the gene functions of M3 liquid samples with that of M1 and M2 liquid samples, with no difference between M1 and M2 liquid samples

Table 2
Functional genes present in the buffalo rumen liquid samples (Values are in percentage).

Subsystems	M1GL	M2GL	M3GL	M1DL	M2DL	M3DL
Amino Acids and Derivatives	8.41	8.55	8.60	8.09	9.03	8.56
Carbohydrates	12.55	11.81	12.54	11.81	12.61	11.53
Cell Division and Cell Cycle	**1.72**	**1.73**	**1.61**	**1.61**	**1.69**	**1.71**
Cell Wall and Capsule	4.90	4.76	4.92	5.21	4.60	4.72
Clustering-based subsystems	15.71	15.84	15.46	16.11	15.54	15.76
Cofactors, Vitamins, Prosthetic Groups, Pigments	5.26	5.06	4.94	5.47	5.24	4.94
DNA Metabolism	5.77	5.80	5.77	5.68	5.63	5.80
Dormancy and Sporulation	**0.39**	**0.37**	**0.35**	**0.37**	**0.35**	**0.33**
Fatty acids, lipids, and Isoprenoids	**1.97**	**1.88**	**1.90**	**2.03**	**1.83**	**1.90**
Iron acquisition and metabolism	0.49	0.60	0.65	0.65	0.63	0.60
Membrane Transport	2.07	2.30	2.38	2.20	2.29	2.35
Metabolism of Aromatic compounds	0.51	0.46	0.48	0.49	0.51	0.48
Miscellaneous	7.58	7.83	7.66	7.77	7.84	7.77
Motility and Chemotaxis	0.38	0.38	0.33	0.36	0.33	0.40
Nitrogen Metabolism	0.98	0.93	0.93	1.04	1.05	0.93
Nucleosides and Nucleotides	3.50	3.71	3.58	3.45	3.68	3.64
Phages, Prophages, Transposable elements, Plasmids	1.93	2.27	1.98	2.10	2.31	1.98
Phosphorus Metabolism	0.37	0.34	0.39	0.39	0.34	0.40
Photosynthesis	0.02	0.03	0.03	0.03	0.02	0.03
Potassium metabolism	0.22	0.18	0.22	0.20	0.18	0.20
Protein Metabolism	11.61	11.49	11.49	11.39	11.10	12.06
Regulation and Cell signalling	5.07	4.95	5.03	5.17	4.83	5.24
Respiration	1.10	1.05	1.08	0.99	0.95	1.09
RNA Metabolism	2.00	2.18	2.27	2.20	2.11	2.22
Secondary Metabolism	0.29	0.32	0.28	0.30	0.30	0.29
Stress Response	1.99	1.90	1.82	1.84	1.86	1.86
Sulphur Metabolism	1.06	0.96	1.06	0.99	1.01	0.85
Virulence, Disease and Defence	2.16	2.31	2.25	2.08	2.16	2.36

Table 3
Functional genes present in the buffalo rumen solid samples (Values are in percentage).

Subsystems	M1GS	M2GS	M3GS	M1DS	M2DS	M3DS
Amino Acids and Derivatives	8.16	8.72	8.52	8.03	8.35	8.54
Carbohydrates	11.22	12.12	12.24	11.40	11.67	11.71
Cell Division and Cell Cycle	**1.84**	**1.80**	**1.71**	**1.86**	**1.82**	**1.79**
Cell Wall and Capsule	4.34	4.57	4.41	4.38	4.23	4.42
Clustering-based subsystems	16.46	15.90	15.76	16.63	16.31	15.81
Cofactors, Vitamins, Prosthetic Groups, Pigments	4.69	4.88	4.73	4.63	4.60	4.80
DNA Metabolism	6.21	5.78	5.78	6.10	6.02	5.58
Dormancy and Sporulation	**0.38**	**0.36**	**0.38**	**0.38**	**0.43**	**0.34**
Fatty Acids, lipids, and Isoprenoids	**1.84**	**1.84**	**1.91**	**1.86**	**1.74**	**1.97**
Iron acquisition and metabolism	0.56	0.63	0.70	0.59	0.64	0.69
Membrane Transport	2.60	2.46	2.70	2.58	2.63	2.76
Metabolism of Aromatic Compounds	0.49	0.48	0.51	0.46	0.49	0.62
Miscellaneous	7.89	7.95	7.90	7.80	7.72	8.07
Motility and Chemotaxis	0.47	0.49	0.41	0.39	0.35	0.44
Nitrogen Metabolism	0.80	0.95	0.92	0.81	0.92	0.98
Nucleosides and Nucleotides	3.73	3.70	3.58	3.79	3.78	3.60
Phages, Prophages, Transposable elements, Plasmids	1.66	1.68	1.50	1.69	1.58	1.45
Phosphorus Metabolism	0.35	0.42	0.40	0.41	0.37	0.44
Photosynthesis	0.02	0.02	0.03	0.02	0.03	0.04
Potassium metabolism	0.17	0.19	0.19	0.18	0.20	0.19
Protein Metabolism	12.16	11.31	11.58	12.12	12.14	11.46
Regulation and Cell signalling	5.33	4.97	5.22	5.32	5.29	5.10
Respiration	1.09	1.11	1.09	1.04	1.08	1.15
RNA Metabolism	2.12	2.15	2.39	2.22	2.31	2.50
Secondary Metabolism	0.28	0.28	0.24	0.22	0.24	0.26
Stress Response	1.97	1.85	1.94	1.90	1.85	1.93
Sulphur Metabolism	0.73	0.99	0.93	0.79	0.87	0.90
Virulence, Disease and Defence	2.44	2.39	2.33	2.38	2.36	2.47

(Table 4), whereas for the Solid samples only M1 Dry solid showed difference with M2 Green and Dry solid and M3 Green and Dry solid samples (Table 5).

3.3. Dormancy and Sporulation

There are multiple classes of Dormancy and sporulation, Cell wall and Capsule and Fatty acids, lipids and Isoprenoids within each functional group. In this study, we used the MG-RAST classification of Dormancy and Sporulation to categorize functional genes. Among the Dormancy and Sporulation genes, sporulation cluster genes were predominant both in liquid (58.97% to 68.98%) and solid (58.46% to 66.51%) samples, which are involved in promoting sporulation to form dormant spores which can survive under stressful conditions (Fig. 3A and B). About 10.12% to 19.20% (liquid samples) and 15.32% to 24.39% (solid samples) of Dormancy and Sporulation genes fell in the category of sporulation gene orphans, which were involved in sporulation; transcription and transcription regulation. Sporulation associated proteins involved in cell cycle; cell-division; septation and sporulation were in the range of 2.04% to 12.08% for liquid samples and 10.57% to 12.44% for solid samples. 7.66% to 13.09% (liquid samples) and 3.07% to 6.88% (solid samples) of genes were associated with spore core dehydration, which is responsible for the maturation of the spore and its dormancy. Also the genes involved in spore germination ranged from 0.43% to 3.23% and 0.44% to 2.42% in liquid and solid samples, respectively (Fig. 3A and B). The statistical analysis using One-Way ANOSIM also revealed significant differences between all the three treatments both for the solid and liquid samples (Tables 4 and 5).

In order to find out which bacterial community contributed to the SEED Subsystem Level 2 category of Dormancy and Sporulation sequence alignment using BLAT integrated in the MG RAST database was carried out which made it possible to look closer at which organisms were associated with the genes assigned to each of the subcategory. Our results revealed that the predominant phylum was of Bacteroidetes followed by Firmicutes and then Proteobacteria in the liquid samples, whereas in the solid

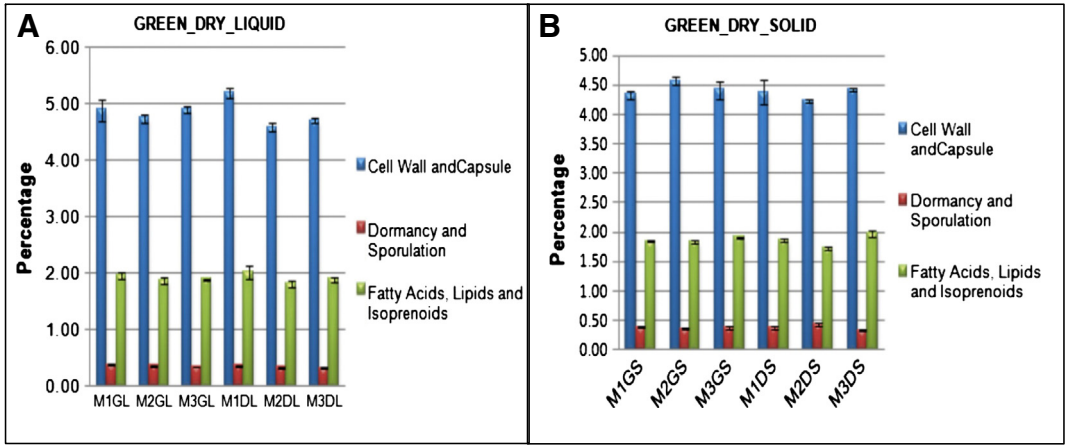

Fig. 2. (A) Chart showing the comparative picture in terms of percentage values for the liquid samples along with their standard errors of mean and (B) chart showing the comparative picture in terms of percentage values for the solid samples along with their calculated standard errors of mean assigned to the three SEED Subsystem Level 1 categories: Cell Wall and Capsule, Dormancy and Sporulation and Fatty acids, lipids and Isoprenoids.

Table 4
Output of the One-way ANOSIM carried out using PAST tool to check the statistically significant difference for the liquid samples at the SEED Subsystem Level 1 for the three categories Cell Wall and Capsule, Dormancy and Sporulation and Fatty acids, lipids and Isoprenoids. (Values in bold denote significant difference between the respective groups).

SAMPLE ID	M1GL	M1DL	M2GL	M2DL	M3GL	M3DL
M1GL	0.00	0.23	0.09	**0.06**	0.12	**0.03**
M1DL	0.23	0.00	0.14	0.14	**0.03**	**0.03**
M2GL	0.09	0.14	0.00	0.26	**0.03**	**0.03**
M2DL	**0.06**	0.14	0.26	0.00	**0.03**	**0.03**
M3GL	0.12	**0.03**	**0.03**	**0.03**	0.00	0.31
M3DL	**0.03**	**0.03**	**0.03**	**0.03**	0.31	0.00

rumen samples the proportion of Firmicutes was more than that in liquid samples (Fig. 4A and B). Clostridia were the predominant taxa contributing to the expression of the genes responsible for spore germination, and sporulation, and sigma factors were associated with sporulation and these mainly comprised of the species like *Clostridium* and *Bacillus* sp.

3.4. Gene assignments to Cell Wall and Capsule

In the category of Cell Wall and Capsule; Capsular and extracellular polysaccharides, Gram-Negative cell wall components and Peptidoglycan Biosynthesis were predominant in all samples. The Cell wall of *Mycobacterium*, Gram-Positive cell wall components, Murein Hydrolases, Peptidoglycan Crosslinking of Peptide Stems, YjeE, Recycling of Peptidoglycan Amino Sugars, UDP-N-acetylmuramate from Fructose-6-phosphate Biosynthesis and tRNA-dependent amino acid transfers are very less abundant (Tables 6 and 7). Metabolic potential of Fatty acids, lipids and Isoprenoids was present in all samples with high abundance. However, Polyhydroxybutyrate metabolism and Triacylglycerols related functions including abundances were very less (Tables 8 and 9). The statistically significant difference for the SEED Subsystem Level 2 categories of Cell Wall and Capsule, Fatty acids, lipids and Isoprenoids and Dormancy and Sporulation is given in Tables 10–15.

3.5. Go Slim analysis

The Gene ontology analysis was performed using GO Slim to identify various functional classes of genes in the buffalo rumen metagenome sample. The GO Slim term associated with Sporulation showed notable variation in the function of the genes across treatments for both the liquid and solid samples with the highest gene function in the 100% roughage treatment and the lowest in the 75% roughage treatment (Fig. 5A and B).

Table 5
Output of the One-way ANOSIM carried out using PAST tool to check the statistically significant difference for the solid samples at the SEED Subsystem Level 1 for the mentioned three categories Cell Wall and Capsule, Dormancy and Sporulation and Fatty acids, lipids and Isoprenoids. (Values in bold denote significant difference between the respective groups).

SAMPLE ID	M1GS	M1DS	M2GS	M2DS	M3GS	M3DS
M1GS	0.00	0.11	0.23	0.65	0.29	0.54
M1DS	0.11	0.00	**0.03**	**0.03**	**0.03**	**0.06**
M2GS	0.23	**0.03**	0.00	0.20	0.86	0.52
M2DS	0.65	**0.03**	0.20	0.00	0.20	0.25
M3GS	0.29	**0.03**	0.86	0.20	0.00	0.83
M3DS	0.54	**0.06**	0.52	0.25	0.83	0.00

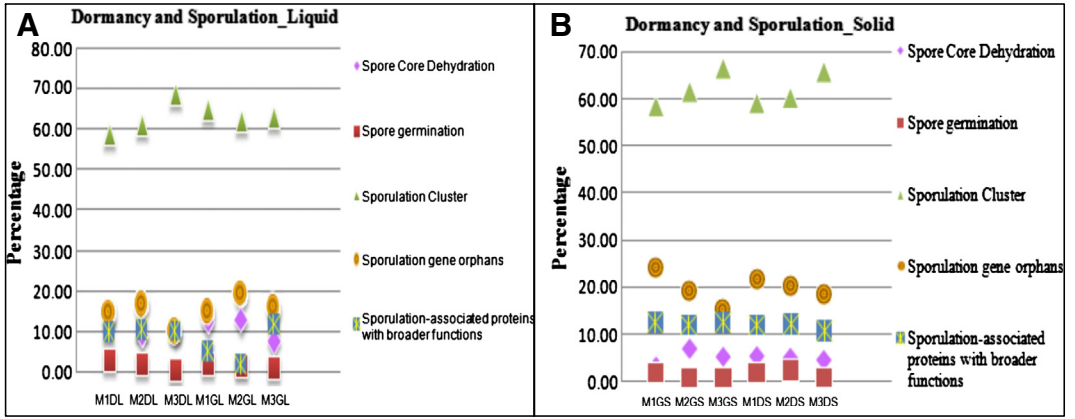

Fig. 3. (A) Percentage values calculated for the liquid samples and (B) percentage values calculated for the solid samples using minimum cut off value of 60% based on the reads corresponding to the SEED Subsystem Level 2 subcategory of Dormancy and Sporulation.

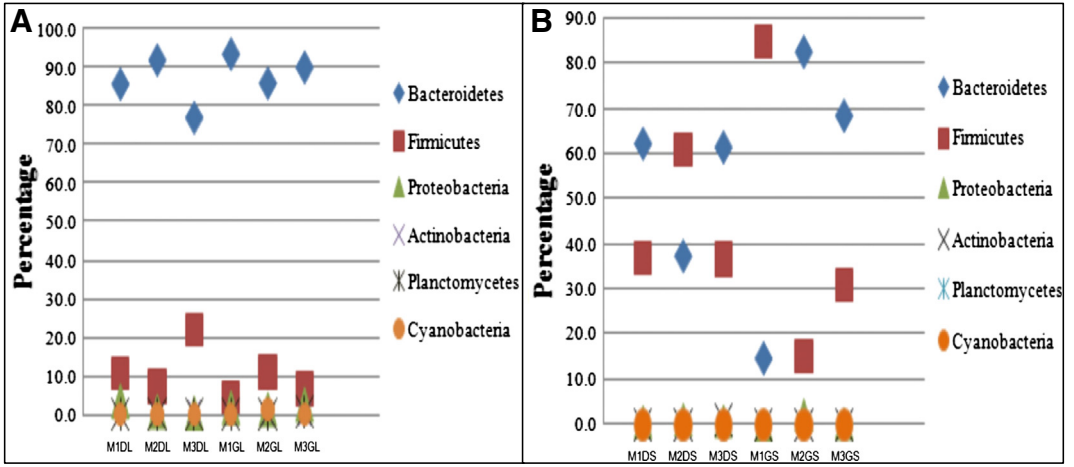

Fig. 4. (A) The phylum level distribution of the organisms in the liquid rumen samples and (B) the phylum level distribution of the organisms in the solid rumen samples for the SEED Subsystem Level 2 category of Dormancy and Sporulation using BLAT using minimum identity cut off of 80%.

Table 6
Percentage values calculated using minimum cut off value of 60% based on the reads corresponding to each SEED Subsystem Level 2 subcategory of Cell Wall and Capsule for the liquid samples.

Subsystems	M1_GL	M2_GL	M3_GL	M1_DL	M2_DL	M3_DL
Capsular and extracellular polysaccharides	**38.12**	**39.12**	**40.06**	**38.92**	**40.18**	**38.72**
Cell wall of Mycobacterium	5.71	5.22	4.96	5.57	5.40	5.75
Gram-Negative cell wall components	**17.02**	**16.26**	**16.29**	**15.70**	**14.81**	**16.38**
Gram-Positive cell wall components	4.62	4.77	4.39	4.22	4.66	4.14
Murein Hydrolases	1.33	1.12	1.09	1.14	1.11	1.18
Peptidoglycan Biosynthesis	**17.64**	**18.12**	**17.96**	**19.72**	**17.37**	**18.49**
Peptidoglycan Crosslinking of Peptide Stems	0.04	0.00	0.01	0.00	0.00	0.00
Peptidoglycan biosynthesis—gjo	5.09	4.92	5.10	5.25	5.16	5.08
Recycling of Peptidoglycan Amino Acids	2.53	1.86	2.13	1.86	2.02	2.01
Recycling of Peptidoglycan Amino Sugars	0.49	0.65	0.51	0.46	0.95	0.54
UDP-N-acetylmuramate from Fructose-6-phosphate Biosynthesis	6.49	6.92	6.43	6.11	7.59	6.82
YjeE	0.92	1.05	1.04	1.04	0.74	0.89
tRNA-dependent amino acid transfers	0.00	0.00	0.01	0.00	0.00	0.01

Table 7
Percentage values calculated using minimum cut off value of 60% based on the reads corresponding to each SEED Subsystem Level 2 subcategory of Cell Wall and Capsule for the solid samples.

Subsystems	M1_GS	M2_GS	M3_GS	M1_DS	M2_DS	M3_DS
Capsular and extracellular polysaccharides	**39.95**	**40.68**	**37.43**	**39.54**	**39.77**	**37.66**
Cell wall of Mycobacterium	4.47	4.60	4.87	4.85	4.42	4.82
Gram-Negative cell wall components	**13.86**	**14.38**	**15.45**	**14.26**	**14.45**	**14.35**
Gram-Positive cell wall components	4.88	4.35	4.16	5.05	4.09	4.50
Murein Hydrolases	1.43	1.12	1.26	1.25	1.48	1.67
Peptidoglycan Biosynthesis	**19.64**	**19.53**	**20.56**	**19.56**	**20.00**	**20.90**
Peptidoglycan Crosslinking of Peptide Stems	0.00	0.01	0.00	0.00	0.00	0.00
Peptidoglycan biosynthesis—gjo	5.05	4.89	5.95	5.23	5.30	5.77
Recycling of Peptidoglycan Amino Acids	1.64	1.85	1.77	1.62	1.70	1.93
Recycling of Peptidoglycan Amino Sugars	0.28	0.53	0.64	0.35	0.37	0.40
UDP-N-acetylmuramate from Fructose-6-phosphate Biosynthesis	7.90	7.29	7.12	7.31	7.71	7.17
YjeE	0.85	0.73	0.77	0.95	0.70	0.83
tRNA-dependent amino acid transfers	0.04	0.03	0.01	0.03	0.01	0.00

4. Discussion

This study demonstrates that untargeted sequencing can be used to detect genes relayed to dormancy and sporulation, Cell wall and Capsule from buffalo rumen metagenomes. The method described for deriving rumen microbiome profiles allows comparison of samples based on the whole population. Using

Table 8
Percentage values calculated using minimum cut off value of 60% based on the reads corresponding to each subcategory of Fatty acids, lipids and Isoprenoids at the Subsystem Level 2 for the liquid samples.

Subsystems	M1GL	M2GL	M3GL	M1DL	M2DL	M3DL
Fatty acids	**52.33**	**47.45**	**51.30**	**50.28**	**49.08**	**50.96**
Isoprenoids	**26.77**	**25.27**	**24.86**	**25.46**	**28.12**	**24.05**
Phospholipids	**17.73**	**22.10**	**18.11**	**21.98**	**17.95**	**20.72**
Polyhydroxybutyrate metabolism	2.91	4.97	5.48	2.08	4.46	4.12
Triacylglycerols	0.26	0.22	0.24	0.20	0.39	0.16

Table 9
Percentage values calculated using minimum cut off value of 60% based on the reads corresponding to each subcategory of Fatty acids, lipids and Isoprenoids at the Subsystem Level 2 for the solid samples.

Subsystems	M1GS	M2GS	M3GS	M1DS	M2DS	M3DS
Fatty acids	**43.61**	**47.49**	**47.11**	**45.21**	**46.45**	**48.19**
Isoprenoids	**25.13**	**25.80**	**23.98**	**24.91**	**26.31**	**23.55**
Phospholipids	**23.78**	**20.91**	**20.93**	**23.03**	**21.33**	**20.92**
Polyhydroxybutyrate metabolism	6.62	5.46	7.49	6.21	5.53	6.80
Triacylglycerols	0.86	0.34	0.49	0.64	0.38	0.54

Table 10
Output of the One-way ANOSIM carried out using PAST tool to check the statistically significant difference for the SEED Subsystem Level 2 categories of Cell Wall and Capsule for liquid samples. (Values in bold denote significant difference between the respective groups).

Sample ID	M1GL	M1DL	M2GL	M2DL	M3GL	M3DL
M1GL	0.00	0.23	0.12	**0.06**	0.11	**0.03**
M1DL	0.23	0.00	0.11	0.14	**0.03**	**0.03**
M2GL	0.12	0.11	0.00	0.29	**0.03**	**0.03**
M2DL	**0.06**	0.14	0.29	0.00	**0.03**	**0.03**
M3GL	0.11	**0.03**	**0.03**	**0.03**	0.00	0.43
M3DL	**0.03**	**0.03**	**0.03**	**0.03**	0.43	0.00

SEED database, the domain-level breakdown of our samples was bacteria, eukaryotes and viruses (Fig. 1). The distribution of sequences from the bacteria was congruent with the distribution of SSU rRNA phylotypes, as reported in the canine intestinal microbiome and cattle faeces microbiome studies (Durso et al., 2011). Phylogenetic potentials of buffalo rumen indicate that the phylum Bacteroidetes was predominant, followed by Firmicutes, Proteobacteria, Actinobacteria and Fibrobacteres in all the diets

Table 11
Output of the One-way ANOSIM carried out using PAST tool to check the statistically significant difference for the SEED Subsystem Level 2 categories of Cell Wall and Capsule for solid samples. (Values in bold denote significant difference between the respective groups).

Sample ID	M1GS	M1DS	M2GS	M2DS	M3GS	M3DS
M1GS	0.00	0.09	0.23	0.88	0.26	0.68
M1DS	0.09	0.00	**0.03**	**0.03**	**0.03**	**0.06**
M2GS	0.23	**0.03**	0.00	0.20	0.92	0.65
M2DS	0.88	**0.03**	0.20	0.00	0.19	0.28
M3GS	0.26	**0.03**	0.92	0.19	0.00	0.97
M3DS	0.68	**0.06**	0.65	0.28	0.97	0.00

Table 12
Output of the One-way ANOSIM carried out using PAST tool to check the statistically significant difference for the SEED Subsystem Level 2 categories of Fatty acids, lipids and Isoprenoids for liquid samples. (Values in bold denote significant difference between the respective groups).

Sample ID	M1GL	M1DL	M2GL	M2DL	M3GL	M3DL
M1GL	0.00	0.20	**0.06**	**0.06**	**0.03**	**0.03**
M1DL	0.20	0.00	0.09	0.12	**0.03**	**0.03**
M2GL	**0.06**	0.09	0.00	0.22	**0.03**	**0.03**
M2DL	**0.06**	0.12	0.22	0.00	**0.03**	**0.03**
M3GL	**0.03**	**0.03**	**0.03**	**0.03**	0.00	0.17
M3DL	**0.03**	**0.03**	**0.03**	**0.03**	0.17	0.00

Table 13
Output of the One-way ANOSIM carried out using PAST tool to check the statistically significant difference for the SEED Subsystem Level 2 categories of Fatty acids, lipids and Isoprenoids for solid samples. (Values in bold denote significant difference between the respective groups).

Sample ID	M1GS	M1DS	M2GS	M2DS	M3GS	M3DS
M1GS	0.00	0.28	0.18	0.69	0.23	0.37
M1DS	0.28	0.00	**0.03**	0.08	**0.03**	0.14
M2GS	0.18	**0.03**	0.00	0.23	0.85	0.34
M2DS	0.69	0.08	0.23	0.00	0.14	0.26
M3GS	0.23	**0.03**	0.85	0.14	0.00	0.66
M3DS	0.37	0.14	0.34	0.26	0.66	0.00

Table 14
Output of the One-way ANOSIM carried out using PAST tool to check the statistically significant difference for the SEED Subsystem Level 2 categories of Dormancy and Sporulation for liquid samples. (Values in bold denote significant difference between the respective groups).

Sample ID	M1GL	M1DL	M2GL	M2DL	M3GL	M3DL
M1GL	0.00	0.20	**0.03**	**0.03**	**0.03**	**0.03**
M1DL	0.20	0.00	**0.03**	**0.03**	**0.03**	**0.03**
M2GL	**0.03**	**0.03**	0.00	0.14	**0.03**	**0.03**
M2DL	**0.03**	**0.03**	0.14	0.00	**0.03**	**0.03**
M3GL	**0.03**	**0.03**	**0.03**	**0.03**	0.00	**0.03**
M3DL	**0.03**	**0.03**	**0.03**	**0.03**	**0.03**	0.00

(Fig. 4A and B). Similar observation has also been reported by Bruce et al. (2008) in cattle rumen and Singh et al. (2012) in buffalo rumen metagenome.

The greatest proportion of gene fragments assigned to known functions was associated with Clustering-based subsystems, carbohydrate and protein metabolisms in similar proportion in all the samples (Tables 2 and 3). The genes belonging to the group of "clustering based subsystems" are frequently found together in multiple organisms for which specific functions are not yet known (Durso et al., 2011). Interestingly, as observed in the Fig. 3A and B, the proportion of genes assigned to the SEED Subsystem Level 2 subcategory of spore core dehydration decreases along the treatments, therefore it could be inferred that during the initial treatment of 50% green and dry roughage, the spores formed were dormant yet with low core water content, no high-energy compounds, and resistant to heat, freezing, irradiation, chemicals, pressure, and desiccation. This ensures long term survival without nutrients. But with the increase in the treatment from 50% roughage to 100% roughage, the core hydration takes place which could be considered ideal for spore maturation and germination. The peptidoglycan (PG) cortex of bacterial endospores is required for the maintenance of spore core dehydration, heat resistance and dormancy (Stephenson and Hoch, 2002; Leggett et al., 2012). Sporulation has been most extensively

Table 15
Output of the One-way ANOSIM carried out using PAST tool to check the statistically significant difference for the SEED Subsystem Level 2 categories of Dormancy and Sporulation for solid samples. (Values in bold denote significant difference between the respective groups).

Sample ID	M1GS	M1DS	M2GS	M2DS	M3GS	M3DS
M1GS	0.00	0.26	**0.03**	**0.03**	**0.03**	**0.03**
M1DS	0.26	0.00	**0.03**	**0.03**	**0.03**	**0.03**
M2GS	**0.03**	**0.03**	0.00	0.65	0.60	0.38
M2DS	**0.03**	**0.03**	0.65	0.00	0.49	0.17
M3GS	**0.03**	**0.03**	0.60	0.49	0.00	0.70
M3DS	**0.03**	**0.03**	0.38	0.17	0.70	0.00

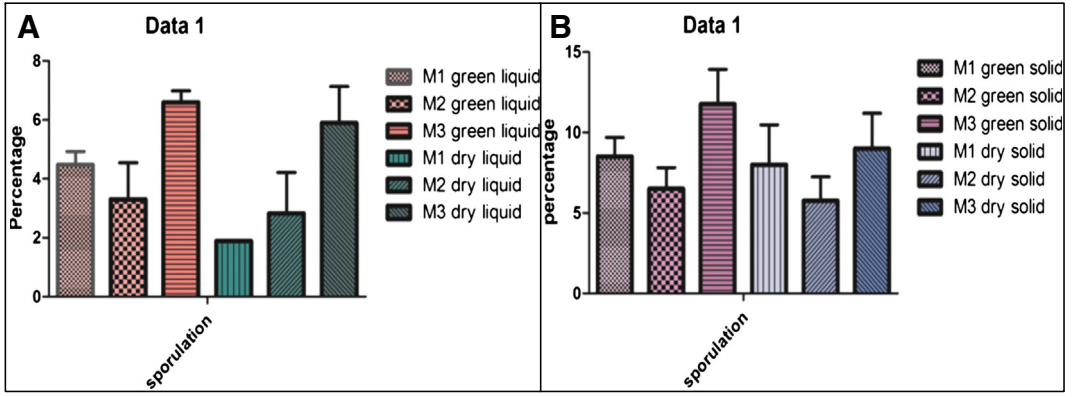

Fig. 5. (A) The graphical representation of the percentage values calculated for the liquid rumen sample and (B) the graphical representation of the percentage values calculated for the solid rumen sample from the output of the Gene Ontology Analysis using GO Slim with respect to the GO Slim term 'Sporulation.'

studied in the model organism *Bacillus subtilis*. Entry into sporulation is governed by the master regulator Spo0A, which is activated by phosphorylation through a multicomponent signal transduction pathway (Gérard et al., 2007) Phosphorylated Spo0A directly regulates (activates or represses) the expression of many genes (Romero-Pérez et al., 2011).

The proportion of functional genes belonging to the subcategory of spore germination increases from the 50% to the 75% roughage treatments considering with respect to the decrease in the values of spore core dehydration. Therefore it is evident that germination of spores takes place across the treatments with the higher proportion of germination occurring in the case of 75% roughage treatment stating that 75% roughage and 25% concentrate ratio is an ideal condition for the germination of the spore.

The results of the sequence alignment using BLAT revealed that the most frequently observed taxa were that of Clostridia and Bacilli which belong to the phylum Firmicutes. In the case of solid sample, Firmicutes and Bacteroidetes complement each other in terms of their concentrations all along the green and dry solid samples (Fig. 4A and B). It has also been reported that Firmicutes phylum mainly consist of Gram positive bacteria (Leggett et al., 2012) and these endospore-forming bacteria belong to the *Bacillus* and *Clostridium* genera. Endospore formation is unique to the low-G–C group of Gram-positive bacteria (Firmicutes). For the most part, it is restricted to the family Bacillaceae and the class Clostridia, but members of the less studied family Veillonellaceae (Sahu et al., 2004; Moto, 2008) also produce endospores.

It could be observed from Fig. 3A and B that the proportion of functional genes associated with sporulation cluster and sporulation gene orphans increased along the treatments both for the green and dry solid and the liquid samples which clearly indicated that sporulation is increasing from 50% roughage to 100% roughage treatments. But as the treatment increased from 75% roughage to 100% roughage sporulation increases leading to unfavourable conditions for the germination of these spores. As reported in literature dormant spores of a number of *Bacillus* species have no detectable biosynthetic or metabolic activity and contain low levels of AT (Brooks and Church, 1957; Warth and Strominger, 1969; KANmdsg., 1970; Setlow., 1970). The lack of enzyme action on endogenous substrates for germination has included low water content and low levels of free metal ions within the dormant spore (Gould and Hurst, 1969; Singh, 1979). A large increase in spore internal water content upon germination has been suggested by other workers on the basis of several types of measurements (Gould and Hurst, 1969; Singh, 1979). Lamont et al. (2011) have identified and described a new spore-like morphotype in *Mycobacterium avium Subsp. Paratuberculosis (PAP)* associated with nutrient starvation. The Gene Ontology analysis using GO Slim also showed a clear variation across treatments in the function of the genes associated with sporulation (Fig. 5A and B), with the highest hits corresponding to the 100% roughage treatment both in the case of green and dry (Solid; Liquid) samples while 75% roughage treatment showed lowest hits with respect to the gene functions associated with sporulation. Metagenomic studies have reported the occurrence of dormancy and sporulation genes in water metagenome (Gomez-Alvarez et al., 2012) and in hydrocarbon-impacted site sample (Smith et al., 2013) by using massive parallel sequencing.

Seed subsystem composition of Cell wall and Capsule of buffalo rumen microbiome indicates the predominance of capsular and extracellular polysaccharides (37.43–40.68%), Gram-Negative cell wall components (13.86–15.45%) and Peptidoglycan Biosynthesis (19.53–20.90%) (Tables 6 and 7). Our results show the presence of Cell wall and Capsule encoding genes in buffalo rumen, reflecting the directional flexibility of structure cell wall in rumen bacteria. High representation of the “cell wall and capsule” subsystem has been reported in bovine rumen (Kav et al., 2012), Yak rumen (Dai et al., 2012) and loris faecal metagenome (Xu et al., 2013).

5. Conclusion

In the present study the bacterial taxa actively playing a role in the Dormancy and Sporulation belonged to Clostridia and Bacilli. Dormancy and Sporulation had statistical significance at both the SEED Subsystem Levels 1 and 2, thus giving an indication that sporulation was promoted across the treatments. The GO Slim results also validated the fact that sporulation increased along the treatments and showed minimum sporulation in the 75% roughage treatment. Also as the values for the functional genes related to spore germination were maximum in the case of 75% roughage treatment, it could be concluded that maximum germination of the bacterial spores is taking place in the second treatment of 75% roughage to

25% concentrate ratio thereby suggesting that it may be an ideal stress-free environment for the microbes residing in the rumen.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mgene.2014.01.005.

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