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REVIEW

Metagenomics and CAZymes in Rumen: A review

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ABSTRACT

Rumen converts lignocellulsic biomass to high quality food by virtue of the diverse microbiota. The composition of rumen ecosystem is shaped up by various factors including diet, individuality, age, geographic region, postfeeding time *etc.* Earlier culture based techniques could study rumen microbes partially for the cultivable microbes. But, now it is known that about 90% of rumen microbes are unculturable and the knowledge that we have till now seems to be meager. With the advent of new technologies as next generation sequencing which study the whole ruminal ecosystem at one time contributed tremendously to our existing knowledge. Approaches like metagenomics, metatranscriptomics *etc.* have made it possible to study the structure and function of rumen microbes in their natural environment. The study of CAZymes revealed that there is an array of hydrolytic enzymes in rumen that perform the deconstruction of fibrous feed material. Also, these enzymes are not only contributed by well known microbes' *viz. Fibrobacter* and *Ruminococcus* but by a very diverse microbiota including *Roseburia, Porphryomonas, Balutia etc.* So, metagenomics has added to our knowledge in many spheres of rumen microbiology, its composition and interactions in rumen. But the problem in this field is that a robust database is not available to compare the data obtained. Much work is required in field of analysis of metagenomic database establishment.

Key words: CAZymes, Fiber, Metagenomics, Rrumen microbes

INTRODUCTION

Rumen is home to complex consortia of microbes and very less is known about the magnitude and direction of interaction of various components of this microbiome. Moreover, only 11% of rumen microbes appear to be culturable (Edward *et al.,* 2004) and those microbes which can be cultured have been studied as pure culture in isolation but in rumen these microbes act in dependent manner. Thus, holistic studies regarding interdependent components of rumen niche and how change in one component affects the other components and finally rumen fermentation needs to be evaluated. There has been a considerable addition to our knowledge in terms of diversity and to some extent functioning of rumen microbiome.

Also, these techniques are biased towards the laboratory cultivable microbes. High throughput techniques like metagenomics provides greater depth of sequencing which produces useful and extensive coverage of the microbial diversity allowing us not only to identify the prevalent 'core' members of the community but also 'rare' community members that could be associated with feeding practices (Shanks *et al.,* 2011). Metagenomics addresses the collective genetic structure and functional composition of microbial community without the bias or necessity for culturing its individual inhabitants (Galbraith *et al.,* 2004).

Rumen metagenomics follows two approaches namely functional metagenomics and computational methagenomics (Puniya *et al.,* 2015). The microbial genes responsible for regulating enzyme production in rumen are expressed in response to various diets and other environmental conditions producing corresponding enzyme. Functional metagenomics include study of collective genome of a microbial community by expressing it in a foreign host (Ekkers *et al.,* 2012) which includes cloning of genetic diversity from rumen in vectors as plasmids, fosmids, cosmids and bacterial artificial chromosome etc followed by expressing it in foreign host as *E. coli* and characterization of desired functional activities in expression libraries using various strategies (Simon and Daniel, 2009). Mining of unexplored and untapped fibre degrading enzymes through this approach may lead to

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revolutions in animal husbandry and industrial sector. Numerous efforts have been made to isolate fibre digesting enzymes of glycosyl hydrolase families as GH3, 5, 8, 10, 13, 43 and 48 (Gruninger *et al.,* 2014; Lee *et al.,* 2012a; Pope *et al.,* 2012).

The current methods are less sensitive in expressing target genes in foreign host and throughput of these screening methods is low (Puniya *et al.,* 2015). But like all new approaches, this approach has many possibilities yet to be explored. The second approach *i.e*. computational metagenomics has made it possible to have deeper insights into structure and functional setup of rumen microbiome which were unachievable earlier. It includes SSU (single sub unit) rRNA approach (16S or 18S) and whole genome shotgun (WGS) approach which provides chance to understand protein range, its metabolic significance and taxonomical assignments of rumen microbes. This will increase our understanding of the structural and functional order of rumen microbes.But these approaches are in infancy and there are many limitations with theses approaches. The major one being that SSU of rumen origin is underrepresented in public database in SSU rRNA approach and inability to assign taxonomy to novel sequences.Whereas in WGS (whole genome sequence) approach and sequences generated by NGS technologies have platform biases, high error rates and short read lengths.

Metagenomics does not reveal how this genetic information is actually expressed in rumen. To study how these genes are expressed and regulated, a new field 'metatranscriptomics' is being explored which aims at studying expression of genes through abundance of mRNA transcripts. Metatranscriptomics gives insight into gene expression and regulation at mRNA level, metaproteomics studies the product *i.e.,* proteins for which these genes are encoded. A step further to this is study of all metabolites of feed and rumen origin (metabolomics) to decipher various metabolic pathways and their interdependence within rumen and how they are affected by varying composition of ruminal consortia. An integrated approach including all 'omics' may give holistic insight into structure and function of rumen microbiome, one of the most complex ecosystem. This review is just touching the tip of the iceberg of this vast knowledge.

METAGENOMIC COGNIZANCE OF RUMEN MICROBIOME

Due to the amazing and unique property of transforming lignocellulosic feed into useful products for human consumption, ruminants, rumen and its microbes have been subject of study since long.

Insights in developing rumen

Lot of research work has done on how rumen microbes are established in rumen since birth and later with development of rumen. Our basic understanding is that rumen is inhabited with microbes within first 24 h of birth (McCann *et al.,* 2014). Bacteria including fibrolytic and methanogens are the first to occupy rumen within first week of life followed by protozoa (Morvan *et al.,* 1994). Earlier studies observed establishment of methanogens within 3-4 days of life (Fonty *et al.,* 1989) but recent studies (Gagen *et al.,* 2012) showed that methanogens can be detected in ovine rumen at 17 h of birth. A detailed study of microbial diversity and functionality in calves (14 and 42 dold) fed similar diet showed that more than 24 prokaryotic phyla and 22 eukaryotic phyla and 8298 Pfam protein families highlighting the diversity richness in pre ruminant calves (Li *et al.,*2011). Further, a total of 156 and 120 genera were found in 14 and 42 d old calves, respectively. Interestingly, rumen of younger calves has more heterogenous rumen microbiome and harbors a greater number of microbial genera than older calves. It might be due to the fact that as rumen develops, opportunistic microbes are eliminated and a certain consortia is developed which favors microbes aiding in fibre degradation.

Jami *et al.* (2013) revealed that *Ruminococcus albus* is detectable at one day of age while another fibrolytic species *F. Succinogenes* developed much later. Presence of fibrolytic microbes much before introduction of solid diet has been observed earlier (Li *et al.,* 2012). The developmental stage of rumen appears to be one of major determinants of microbial setup in rumen and the rumen microbiome becomes more

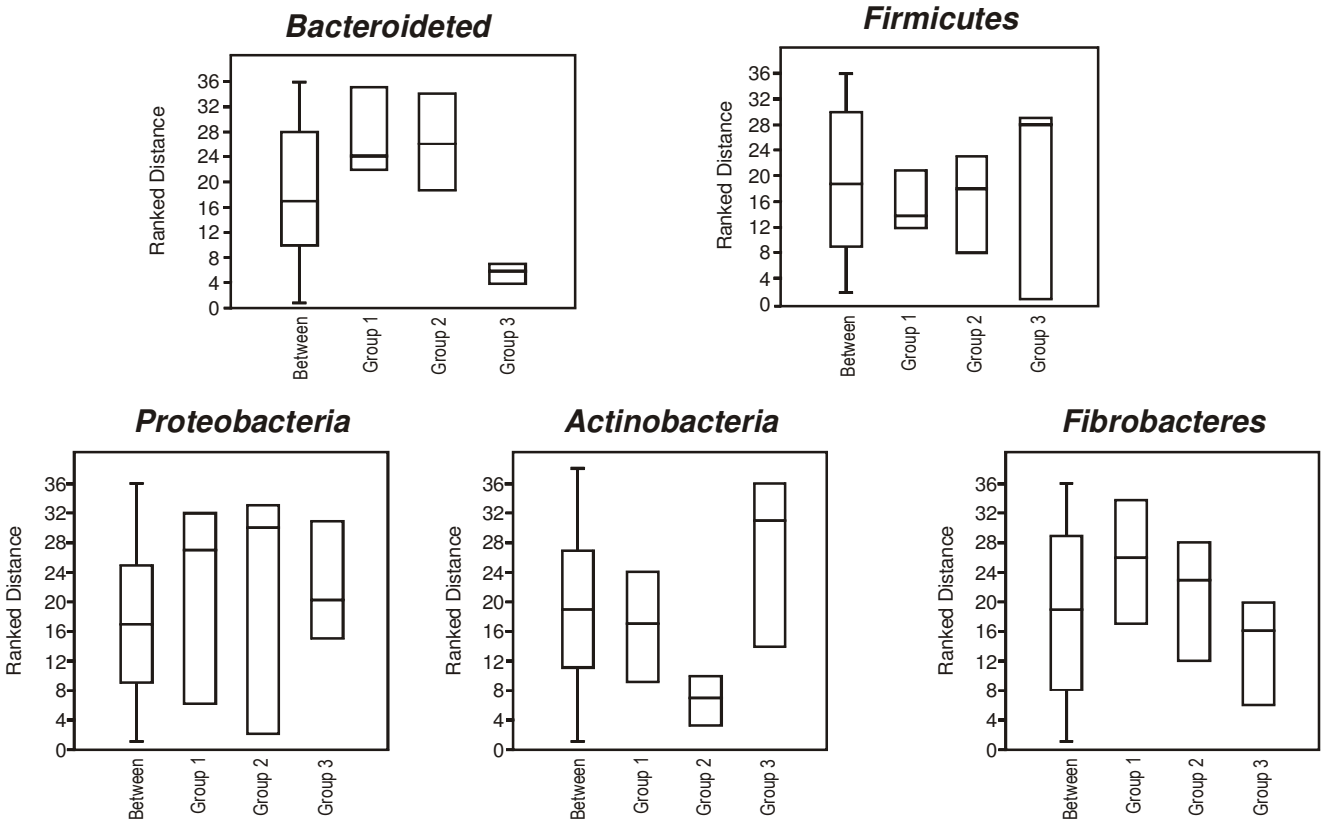

Fig.1. Box plot indicating mean of ranked distances for most abundant five rumen phyla in buffaloes fed three levels of TDN. Group 1, 2 and 3 indicate 70, 85 and 100 % TDN fed groups. Boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentile) and the horizontal line defines the median (Source: Kala *et al.,* 2017a).

homologous with increased diversity as the age of the calf advanced.

Revealations from nutritional interventions

Pioneer studies on rumen metagenomics (Brulc *et al.,* 2009) using next generation sequencing (NGS) indicated that individuality of animal may be dominating over other factors (as diet, environment) in establishing rumen microbial composition. In above study fibre adherent portion microbiome in which enrichment (66 *vs.* 8% abundance) in *Gamma proteobacteria* at the expense of *Firmicutes* and *Bacteroidetes* was observed in third animal as compared to other two animals. It appeared that third animal had not adapted to experimental diet, which may be due to individuality of animal. Similar observation were made by Kala *et al.* (2017) wherein buffaloes fed varying TDN diets showed great variation in rumen microbial abundance which might be attributed to individuality of buffaloes in group fed similar diet.

There are many factors (diet, individuality of animal, type and time of sample, processing of sample, sequencing technologiy *etc.*) which need to be considered during metagenomic studies.

Distribution of microbes in rumen

Metagenomics of rumen microbes has provided the opportunity of studying rumen ecosystem in its enteriety considering all the cultivable and non-cultivable microbes. In rumen, bacterias are the most abundant domain followed by eukaryota, archea virus and phages (Kala *et al.,* 2017a). Baceriodetes and firmicutes have been reported to be the most abundant phyla in the rumen of beef cattle, Sika deer and buffaloes irrespective of diet or treatment (Durso *et al.,* 2011; Mao *et al.,* 2015; Nardi *et al.,* 2016; Kala *et al.,* 2017a). In buffalo rumen, Bacteroidetes and firmicutes followed by proteobacteria, actinobacteria and fibrobacteres

were predominant bacterial phyla (Fig. 1) all the experimental animals except two animals, one from 85% TDN group and another from 100% TDN group where fibrobacteres were higher than actinobacteria (Kala *et al.,* 2017a) which may be individual animal variation. PCA plots for diversity analysis at phylum level revealed that buffaloes supplemented 85% TDN diet had the most diverse rumen microbiome followed by 100 and 70 % TDN in diet (Fig 1) and that some of the phyla were common to all the experimental animals.

At the level of genera, *Prevotella* is reported to be the most dominant bacteria followed by *Bacteroides* and *Clostridium* (Singh *et al.,* 2012; Kala *et al.,* 2016b). Many studies observed similarity in predominance of the major genera in bovine rumen (Li *et al.,* 2014; Mao *et al.,* 2015; Kala *et al.,* 2017a) irrespective of different dietary interventions. Most of metagenomic studies have reported a considerable proportion of rumen microbes to be unidentified or lesser known. Kala (2017) also observed that about 50% sequences of metatranscriptomic libraries of buffalo rumen were devoted to sequences with less than 1% abundance, unknown or unassigned sequences and were termed as others but this cluster of 'others' might have microbes of importance in rumen function and need to be explored further.

Abundance of microbes in rumen could be classified with *Firmicutes* and *Bacteroidetes* forming major chunk of rumen bacterial population. Kala (2017) reported that distribution of rumen phyla showed shift in abundance with respect to three different levels of TDN (70, 85 and 100%) in diet, showing highest diversity for 85 % TDN group (Fig. 2). However, Menezes *et al.* (2011) reported that the phylum separation was more evident for sample type (solid and liquid portions) rather than separation between diets (Menezes *et al.,* 2011). They observed that *Fibrobacteres* and *Spirochaetes* were more prevalent in solid phase whereas *Actinobacteria* was more abundant in liquid phase. The overall dissimilarity was higher for solid and liquid fractions (14.9%) rather than diets (10.5%). They revealed that bacteria and archea were affected by both diets and rumen phase (solid or liquid). Fouts *et al.*

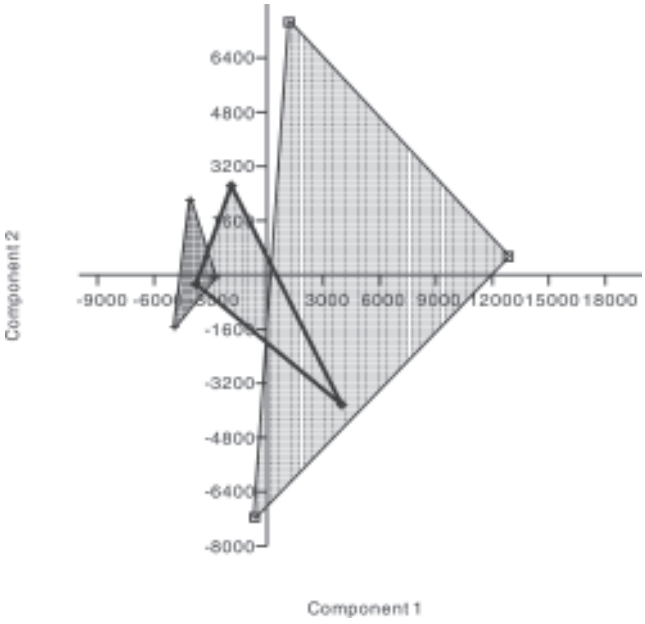

Fig. 2. PCA plots representing diversity shifts at phylum level in buffalo rumen with supplementation of TDN levels. The experimental groups are represented by colors red (70 % TDN), purple (85 % TDN) and green (100% TDN).

(2012) reported that 80% of species levels OTU were dominated by *Clostridales, Bacteridales, Erysipelotrichales* and unclassified TM7 in cows fed similar diets. *Prevotella* and *Taneralla* were abundant in liquid portion while *Butyrivibrio* and *Balutia* were higher in solid portion but no statistical difference was observed in solid and liquid portions. Analysis of cross domain correlations suggested far greater interdependent rumen microbial diversity. Kittelmann *et al. (*2011) showed rhatcommunity compositions of ciliate population in cattle were observed to be influenced by diet whereas diet effects in deer and sheep were weaker than the animal-to-animal variation. Further. it was revealed that New Zealand cattle contained A-type communities with most sequences closely related to those of the genera *Polyplastron* and *Ostracodinium* whereas deer and sheep harboured B-type communities, with the majority of sequences belonging to the genera *Epidinium* and *Eudiplodinium* (Kittelmann *et al.,* 2011).

It is still not very clear as to what affects the

composition of rumen microbiome in ruminants but diet and individuality of animals seem to play major role along with other factors as species, geographic region, age of animal *etc.* Methanogenic archaea in the rumen have not been very well documented and not much information was available as compared to other anaerobic habitats making rumen environment highly selective. In buffaloes, we found about 22 genera along with some unidentified groups thus unfolding the richness of rumen methanogen community structure upto some extent (Kala *et al.,* 2017c). Domain archea is further divided to phylum, Crenarchaeota, Euryarchaeota, Thaumarchaeota, Nanoarchaeota. Most of the methanogenic archea belonged to Euryarcheot including *Methanobrevibacter* which has been reported to be the most abundant methanogen in rumen (Zhu *et al.,* 2017; Kala *et al.,* 2017a) and is responsible for majority of methane production in ruminants. The enteric methane production was said to be positively correlated to abundance of rumen methanogens (Ross *et al.,* 2013; Wallace *et al.,* 2015) but some researchers reported that enteric methane production is dependent on transcription of methanogenic pathways (Shi *et al.,* 2014) or composition of methanogenic consortium in rumen ((Danielsson *et al.,* 2017).

Earlier, *Fibrobacter* and *Ruminococcus* were known to be most abundant rumen microbes (Kala *et al.,* 2017b) and their role in fiber degradation is well known but their low abundance in metagenomic studies (Kala *et al.,* 2017a; Lim *et al.,* 2013) pointed that there are other rumen bacteria playing crucial role in fiber degradation. Stevenson and Weimer (2007) also reported that the key fibrolytic bacteria *viz.*, *R. flavefaciens, R. albus* and *F. succinogenes* represented only ~2% of ruminal bacterial 16S rRNA. In our studies also, microbes like *Paludibacter, Blautia, Porphyromonas* and *Roseburia* contributed sizably to the CAZymes involved in fiber digestion. Many studied have reported *Bacteroides* as one of the most abundant bacteria in buffalo rumen after *Prevotella*. Naas *et al.* (2014) reported that *Bactreoides* has celluloytic activity of and the presence of polysaccharide utilizing loci (PUL, a system of lignocellulosic feed utilization other than extracellular and cell bound cellulosomes) in their genome further strengthens the presence of an alternate mechanism of fiber utilization in rumen.

Core rumen microbiome

The rumen microbiome is responsive to diet, developmental stages, genetics and environmental factors which may alter rumen microbial composition substantially. But it may be possible that a set of core genus/OTU is present across all ruminant species which is involved in basic rumen functioning. This may be said as core microbiome which may deepen our understanding of basic structure and function of rumen microbiome. The core rumen microbiome of bovine rumen microbiome, developing as well as mature was reported to consist of 8 phyla, 11 classes and 15 families (Wu *et al.,* 2012). These 8 phyla included Bacteroides, Firmicutes, Proteobacteria, Spirochaetes, Fibrobacteres, Verrucomicrobia, Synergistetes and Actinobacteria in descending order of relative abundance. A comparative study of microbial community composition of bovine, caprine and ovine rumen showed that a total of 22 phyla and 94 families were detected in rumen microbiome of cattle, goat and sheep (Kim *et al.,* 2011) with mean number of phyla per animal rumen 16.8, 11.8 and 16.9 for cattle, goat and sheep, respectively. Moreover, the relative abundance of the core microbiome varied as per species of ruminants. The author has also observed 46 and 70 phyla common to nine metatranscriptomic libraries in two different experiments in buffaloes fed different TDN (Fig. 3) or blend of essential oils (Kala, 2017).

Metanalysis of rumen microbial diversity of 16S rRNA gene sequences of rumen origin deposited in RDP database identified 19 phyla and, 5271 and 942 OTUs for rumen bacteria and archea, respectively. Of these 19 phyla, Firmicutes, Bacteriodetes and Proteobacteria were most predominant.

Rumen plasmidome

Till now, focus of rumen microbiology has been on more known rumen bacteria, archea and fungi but now it is being diverted to other less studied components as 'plasmidome' and 'virome' of rumen. These small components are major factors deciding

adaptation of rumen microbiome during changing conditions of rumen and they should be taken to consideration when studying a complex system as rumen. Plasmids have not been studied much till now because copy numbers of plasmids are very low per bacteria and it is difficult to differentiate them from chromosomal DNA. Brown Kav *et al.* (2013) reported a method to enrich rumen plasmid DNA. Plasmids may affect the representation of important phyla in rumen. *Proteobacteria* account for 20% of rumen plasmid sequences whereas its abundance appears to be significantly lower (approx. 5%) as per 16 S approach (Brown Kav *et al.,*2012).These plasmidome share similarities with rumen metagenome and display higher functional representation of cell wall and capsule, cofactors and vitamins *etc.* This hints at possible lateral gene transfer between rumen microbes (Puniya *et al.,* 2015).

Rumen virome

Due to lack of conserved protein and genes as 16S rRNA, rumen viral diversity has not been studied much. In a recent study (Ross *et al.,* 2013), 14 putative viral sequences over 30 kbp are identified. Rumen virome of cows housed together and fed similar diets displayed similar taxonomical virome profiles than non -cohabitated animals indicating rumen viromes might be functionally conserved. This also infers that rumen virome are related with transfer of antibiotic resistance, control bacteria population dynamics; regulate protein metabolism and animal nutrition. These factors may finally affect the resilinence of rumen microbes to alteration of rumen environment and host immunity.

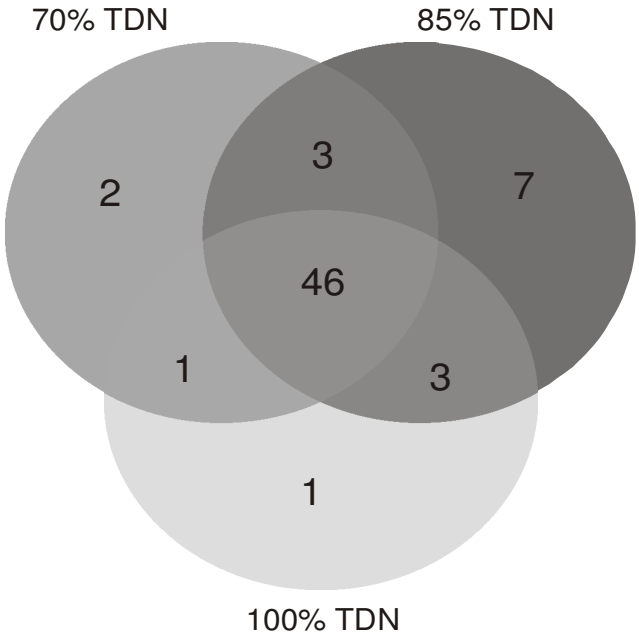

Fig. 3. Venn plot showing shared and unique phyla in rumen of buffaloes fed 70, 85 and 100 % TDN. All taxa present within each group are plotted (As per Kala *et al.,* 2017a).

Carbohydrate active enzyme profile

Rumen microbes produce a vast array of hydrolytic enzymes which are responsible for deconstruction of structural plant polysaccharides. Activity of hydrolytic enzymes in rumen is documented (Agarwal *et al.,* 2000, Patra *et al.,* 2010) to some extent. Functional metagenomics has been used to identify majority of these enzymes of biotechnological interest using specific substrates. This approach was pioneered by Ferrer *et al.* (2005) who detected 9 endoglucanases, 12 esterases and 1 cyclodextrinase from a dairy cow rumen metagenomic library. Functional properties of complex ecosystem as rumen needs to be understood as closely related microbes may have different functions and metabolic characteristics (Achenbach and Coates, 2000; von Mering *et al.,* 2007). Arumugam *et al.* (2011) suggested that functional biomarkers are more robust than phylogenetic biomarkers for identifying enterotypes associated with host phenotype characteristics. In agreement to this, we also found in our studies that CAZymes which are considered important for fiber utilization in rumen (GH2, 3, 5, 94) had a larger contribution from rumen microbes of lesser known origin. Microbes as *Paludibacter, Roseburia, Balutia* and *Porphyromonas* were some of the microbes contributiong sizably to CAZymes in buffalo rumen (Kala *et al.,* 2017a).

The CAZyme database gives information regarding the gene sequences encoding for carbohydrate utilizing enzymes mainly glycoside hydrolases (GHs) which hydrolyze the glycosidic bonds of polysaccharides, carbohydrate esterases (CEs) and break the ester bonds between lignin and carbohydrates,

cellulose binding module (CBMs) involved in enzyme substrate binding, pectin lyase related pectinolysis, glucosyl transferase involved in transfer of glucosidic bonds (Kala *et al.,* 2016). Earlier, it was not possible to mine this Carbohydrate Active Enzyme (CAZymes) profile of rumen at such greater depths but with advent of NGS and meta-transcriptomics, it became possible to identify putative CAZymes of rumen origin. Functional screening and sequencing of fosmid libraries from Svalbard reindeer rumen revealed CMCase positive clones affiliated to the abundant SRM-1 clade that lacked known endoglucanases inferring the possibility of novel cellulolytic mechanisms that are yet to be characterised. This approach also identified *Bacteroidetes*-affiliated GH5-linked PULs (Pope *et al.,* 2012). Similar cellulose linked PULs were found in the cow and buffalo rumen and macropod foregut microbiomes indicating that these structures might be involved in adaptation to growth on cellulose by *Bacteroidetes* species. The solid and liquid mass of rumen together form heterogenous rumen content harbouring microbes producing CAZymes (Hobson, 1997) and the whole rumen content forms ideal sample for mining CAZymes.

Patel *et al.* (2014) found that the proportion of sequences showing > 90% identity to known CAZymes was very less which indicated the presence of novel enzymes which could be further explored for their activity. Among known CAZymes, only GH5 and 9 accounted for about 1.81% of all the glycoside hydrolases of cellulases representing families and GH 10, 11, 26 and 28 collectively represented a total of 4% of all hemicellulases GHs. In our study (Kala *et al.,* 2017a), the major contributors to CAZyme data were GH families like GH 2,3,5,9,29 and 39 (cellulase) and GH 43,51,78 (hemicellulase).Also, those GH families which are considered more important for fiber degradation sourced from a variety of rumen microbes indicating their source diversity. This diversity of source of enzyme might be a mechanism to counter situations when some particular species of rumen microbes are adversely affected in conditions like stress or diet change but the essential functions of rumen fermentation need to be carried using other microbial sources. This may answer why it is difficult to change rumen fermentation pattern by changing diet of experimental animals. The relative proportion of each class of the CAZymes increased with increase in the roughage proportion in the diet indicating enrichment of microbial for utilization of complex plant polysaccharides in rumen (Patel *et al.,* 2014).

The most abundant CAZymes in rumen metagenome are oligosaccharide degrading enzymes (Wang *et al.,* 2013) which include GH 2, 3, 10, 11,5, 43, 51, 95, 36 *etc.* in which GH2, 3, 43 are the most abundant family. Higher abundance of oligosaccharide degrading enzymes in rumen is quite expected as the hemicelluloses chain of dietary origin has variety of side chains and to break the side chains diverse enzymes will be required in rumen. A comparison of glycoside hydrolase and cellulosome functional genes revealed that in the rumen microbiome, initial colonization of fiber appears to be by organisms possessing enzymes that attack the easily available side chains of complex plant polysaccharides (families GH2 and GH3) and not the more recalcitrant main chains especially cellulose (GH5 and GH9). For example, the metagenomes displayed a diversity of enzymes that digest the side chains of cellulose, hemicelluloses and pectins and oligosaccharides but the number of enzymes devoted to the hydrolysis of the main chain of these polymers is very small (Brulc *et al.,* 2009). CBM also plays very crucial role in fiber utilization as CBM class helps in attachment of substrate and enzyme.Moreover CBM37 was observed to contribute solely from *R. albus* in rumen and *R. albus* is well known for fiber utilization (Ezer *et al.,* 2008). White *et al.* (2014) also reported that CBM37 binds to numerous types of polysaccharides and is associated with several enzymes including xylanase and esterase of plant cell wall catalytic modules.

CONCLUSIONS

Metagenomics has broadened horizons of our knowledge in revealing the lesser known facts of rumen microbiome. This approach is addressing some

long unanswered questions. The majority of rumen bacteria as observed through metagenomic studies are *Bacteroidetes, Firmicutes* and *Proteobacteria*. Among archea, *Euryarcheota* is major group. But in all the studies, a large number of sequences are unclassified which form major proportion of rumen microbiome. So, urgent focus is required on characterizing and classifying these unknown sequences. Further, rumen microbiome composition may be affected by species, genetics and individuality, type of sample (solid or liquid). Also, the presence of fiber degrading microbes in day old rumen indicates possibility of introducing fibrous diet at younger age. But further conformational studies are required. There could be alternate methods of fiber degradation in rumen as observed by PUL studies which might be explored further as tools for enhancing fiber degradation. There could be a possibility that the predominant and 'main' rumen microbes are not the only drivers of rumen functions but other 'rare' and less represented microbes might decide dimension and direction of rumen fermentation. Also, the CAZyme family studies indicated a diversity of microbes which contribute rumen enzymes, hence indicating involvement of other less represented microbes. Though metagenomics has added to our knowledge in many spheres of rumen microbiology but to put forth concrete facts for correlating rumen fermentation and rumen microbiome, more work is required in fields of analysis and database establishment.

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