Chapter 5

Phylogenetic and Functional Diversity of Faecal Microbiome of Pack Animals

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.69252

Abstract

The present chapter describes the microbial diversity of faecal microbiomes of pack animals. The sequencing data generated through ion semiconductor sequencing technology were analysed using EBI metagenomics and MG-RAST server tools. Bacteria were the major domain in all the pack animals. At the phylogenetic level, Firmicutes was the major phylum. Clostridiales was the major order. *Ruminococcus flavefaciens* was the major species in camel, whereas the top-most species existing in Equidae family was *Streptococcus equinus*. Among the 28 major functional categories, protein metabolism functionality was dominant in pack animals. The genes associated with protein processing and modification as well as for protein folding are higher in mules and in camel they are lowest. Central carbohydrate metabolism was the major functional group under carbohydrate metabolism in pack animals. Variation in the amino acids and its derivatives was seen in pack animals. Genes associated with proline and 4-hydroxy prolines were present in Equidae family only. Clustering using ward with Bray-Curtis distance matrix for the functional categories showed that donkey and mule are most closely related and clustered with the horse metagenome.

Keywords: Camelidae, Equidae, faecal microbiome, taxonomic, functionality

1. Introduction

The pack animals, namely camel, horse, mule and donkey are traditionally regarded as animals for transportation/draught. Among pack animals, dromedary camel is a pseudo-ruminant and a foregut fermenter, while equidae members are non-ruminant hindgut fermenters. Anaerobic habitats have existed continuously throughout the history of the earth, the gastrointestinal tract being a contemporary microniche [1]. The microbial community inhabiting

the gastrointestinal tract is represented by all major domains of microbes, including Bacteria, Archaea and Eucarya [2] as well as viruses (bacteriophage), and characterized by its high population density, wide diversity and complexity of interactions which play a vital role in the normal nutritional, physiological, immunological and protective functions of the host animal.

Literature shows [3–6] that 1–5% of the microbial diversity can be known through cultivation techniques. Over a period of time, advances from a culture-dependent to culture-independent technologies have revolutionized understanding the microbial ecology. Metagenomics or culture-independent genomic analysis helps to understand the biology of uncultured bacteria, archaea and viruses which can unveil the genetic diversity, population structure and ecology in particular environmental niche [7]. As sequencing costs continue to diminish, the breadth of metagenomic research increases. The sequencing technology and bioinformatics have enabled the molecular characterization of gastrointestinal microbial populations in livestock. Metagenomics helps in visualizing the complex microbial communities which have impact on the health of animals and human and global biogeochemical cycles [8–10]. Next-generation sequencing technologies were being used to characterize the microbial diversity and functional capacity of a range of microbial communities in the gastrointestinal tracts of several animal species [11–18]. Understanding the genetic composition of faecal microbial communities does have implications on food and water safety and animal faeces can also harbour human pathogens. The personal genome machine (PGM) platforms provide a low-cost, scalable and high-throughput solution for studying the microbial community structure and function analyses [19].

2. Methodology

Upon the ethical committee approval, faecal samples from pack animals (camel, horse, mule and donkey) were collected from rectum and immediately placedon ice and stored at ⁻80°C till further DNA extraction; 250 mg of faecal material was subjected for lysis and DNA was extracted by the using QIAmp DNA stool mini kit (Qiagen, USA). The DNA purity and concentration was analysed by spectrophotometric quantification and gel electrophoresis. Enzymatic fragmentation was done to yield fragments of 280–300 bp size. Later library construction followed by emulsion polymerase chain reaction (ePCR) was done. The recovered ePCR product was loaded onto Ion torrent PGM 316 chip for sequencing as per manufacturer's instructions on Ion Torrent PGM. Generated data were uploaded on MG-RAST (the Metagenomics RAST) server. MG-RAST server [20] is an automated platform for the analysis of microbial metagenomes to get the quantitative insights of the microbial populations. Metagenomic comparisons were made with the yet-to-publicize metagenomic data sets of camel (4513857.3), horse (4514961.3), mule (4514940.3) and donkey (4514220.3) on MG-RAST Server. The Post QC data were also submitted to EBI metagenomics [21] in the projects camel (ERS631575), horse (ERS631759), mule (ERS631825) and donkey (ERS631580) for comparing the microbial diversity which are yet to be publicized. The maximum e-value of 1e⁻5, minimum per cent identity of 80 and minimum alignment length of 50 bp for 16SrRNA taxonomy and 30 bp for functionalities were applied as the parameter settings in the analysis. Clustering was performed using Ward's minimum variance with Bray-Curtis distance matrix for normalized values on MG-RAST analysis was done.

3. Faecal microbial diversity

3.1. Diversity of the camel faecal metagenome sequences

The summary of the sequencing datasets uploaded on MG-RAST is shown in **Table 1**.

3.2. Taxonomic classification

The phylogenetic data revealed bacteria as the major domain in all pack animals. Firmicutes was the major phylum. A total of 22–31% of reads were unassigned bacterial phylum. In camels, higher Firmicutes to Bacteroides ratio of 3.8 was observed, whereas in horse, mule and donkey the ratio was 1.5, 1.6 and 1.7, respectively. The difference in the microbial diversity at the phylum level may be due to the variations in digestive physiology of camels and equines. **Figure 1** represents the per cent abundance of operational taxonomic units (OTUs) at phylum level. Fusobacteria and Fibrobacteres phyla were exclusively observed in donkey, whereas WPS-2, Actinobacteria, and Elusimicrobia were found exclusively in mule, camel and horse, respectively. In mules, >10% of the reads were assigned to Verrucomicrobia phylum. In human beings, the *Firmicutes*/*Bacteroidetes* ratio undergoes an increase from birth to adulthood and is further altered with advanced age [22]. *Verrucomicrobia* is a universally distributed phylum and first observed in freshwater [23]; this phylum has already been discovered in termite gut, human intestines and sea cucumbers as well as in very extreme environments [24]. All the pack animals showed this phylum with high abundance in mules. Comparative analyses of 16S rRNA gene sequences prepared from the foregut contents of 12 adult feral camels in Australia fed on native vegetation also observed that the majority of bacteria were affiliated to phylum Firmicutes. The remaining phyla were represented by Actinobacteria, Chloroflexi, Cynophyta, Lentisphaerae, Planctomycetes, Proteobacteria and Sphirochaetes [25]. The taxonomic analysis of metagenomic reads indicated Bacteroidetes (55.5%), Firmicutes (22.7%) and

Metagenome id		Camel	Horse	Mule	Donkey
Post QC data	bp count (bp)	55,194,766	43,405,015	81,917,010	41,499,354
	Sequence count ORF's	385,464	321,769	561,418	275,682
	Mean sequence length (bp)	143 ± 63	134 ± 61	145 ± 63	150 ± 65
	Mean GC per cent (%)	46 ± 10	46 ± 9	47 ± 10	47 ± 9
Predicted	Protein features	306,905	256,458	461,826	233,866
	rRNA features	73,473	55,421	95,936	43,902
Identified	Protein features	132,735	104,681	177,488	96,095
	rRNA features	843	523	910	854
	Functional categories	80,877	64,961	109,704	58,412

Table 1. Summary of analysed data of faecal metagaenomes of pack animals.

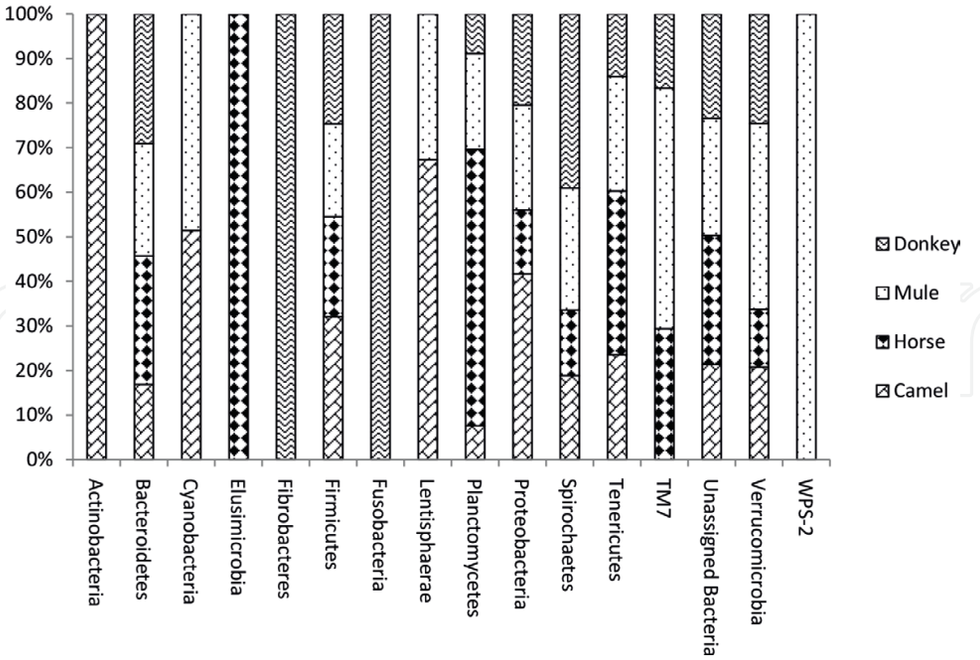

Figure 1. Per cent abundant OTUs of different phyla in pack animals.

Proteobacteria (9.2%) phyla as predominant camel rumen taxa and *Bacteroides* species dominated the camel rumen metagenome [26]. But in the faecal metagenome, Firmicutes was the major phylum in camel. The alteration in the part of the digestive tract does have influence on its microbial diversity.

The phylogenetic resolution at order, genus and species was assigned a maximum e-value of 1×10^{-5}, a minimum identity of 80% and a minimum alignment length of 50 bp using M5RNA data base within MG-RAST. Microbial diversity at the order level revealed more microbes in Clostridiales (>50%) followed by Bacteroidales (>10%) in camel. In horses, Clostridiales (38.2%) followed by Lactobacillales (22.9%) and Bacteroidales (11.4%) were the predominant orders. In mules and donkeys, Clostridiales (39.9 and 43.2%) followed by Bacteroidales (16.2 and 17.5%) and Lactobacillales (8.5 and 14.5%) were the predominant orders. At the genus level, Clostridium was the major organism in mule and camel, while Streptococcus was most abundant in horse and donkey. The top-most genera (>1%) were shown in **Figure 2a–d**. In camels, *Ruminococcus flavefaciens* is the most abundant species and in all equidae members *Streptococcus equinus* is the major organism at species level.

3.3. Predicted gene functions

The data were analysed using SEED subsystem within MG-RAST. An overview of the predicted functions of genes sequenced from pack animals was presented in **Table 2**. Twenty-eight functional categories were assigned with maximum per cent of genes assigned for protein metabolism in all pack animals (>10%). The study on camel rumen functional analysis revealed that clustering-based subsystem and carbohydrate metabolism were the most abundant SEED subsystem representing 17 and 13% of camel metagenome, respectively [26].

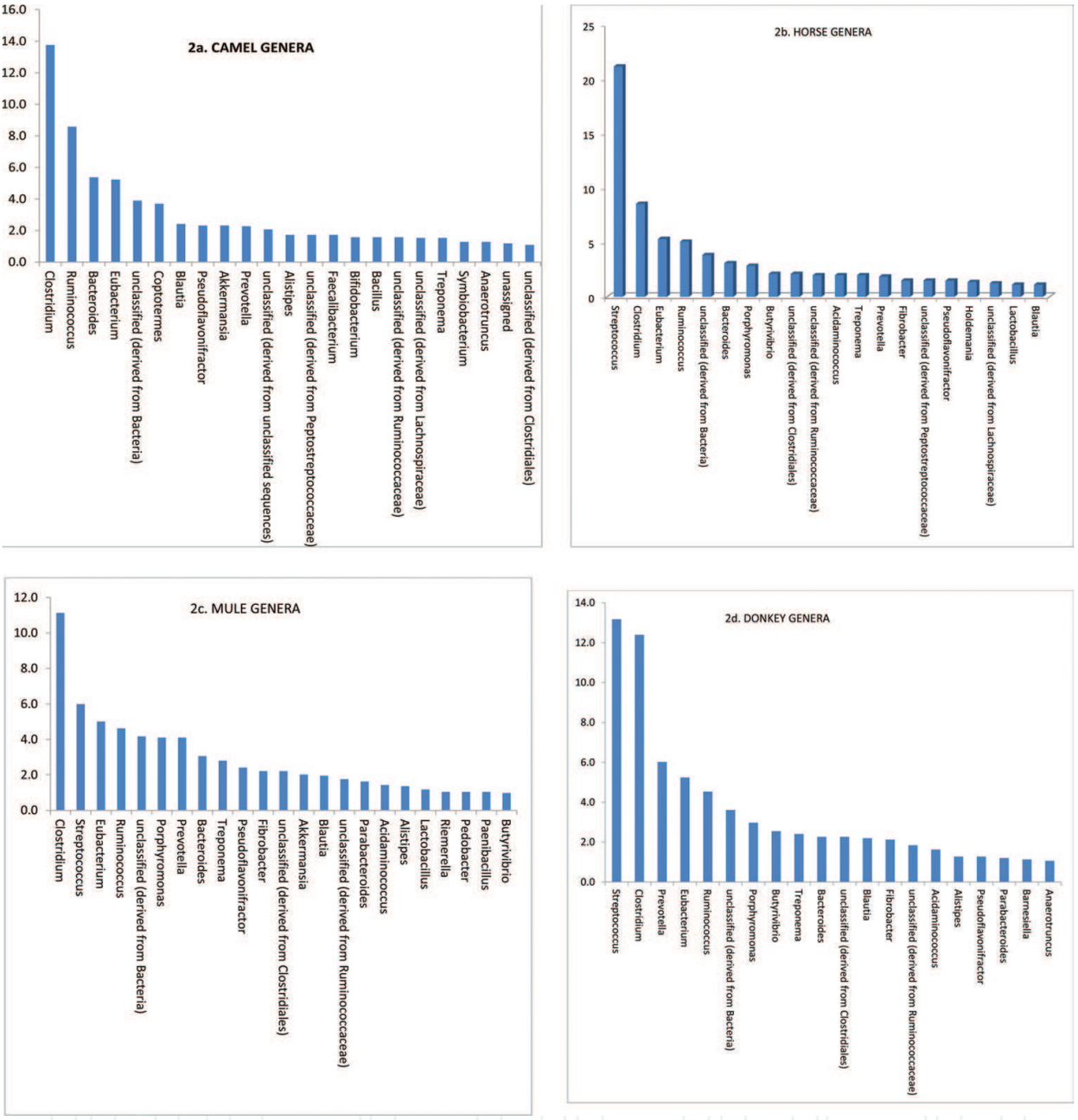

Figure 2. (a–d) Per cent abundance of different genera in pack animals (>1%). The data were compared to M5RNA using a maximum e-value of 1e−5, a minimum identity of 80% and a minimum alignment length of 50 measured in bp for RNA databases.

3.3.1. Protein metabolism

In protein metabolism, the sub-category of genes associated with protein biosynthesis showed high abundance in all pack animals (**Figure 3**). Among the genes associated for protein metabolism, the genes of protein degradation were highest in horses (12.9%) and lowest in mules (1.2%). In mule, a high percentage of genes for protein processing and modification as well as for protein folding were observed and in camel they were lowest.

Functional categories	Camel	Horse	Mule	Donkey
Amino acids and derivatives	7.9	4.7	3.9	4.3
Carbohydrates	8.7	8.8	10.6	8.9
Cell division and cell cycle	1.3	0.7	1.2	0.9
Cell wall and capsule	2.3	1.8	3.0	2.6
Clustering-based subsystems	7.4	8.7	7.2	6.4
Cofactors, vitamins, prosthetic groups, pigments	3.0	3.8	3.0	2.3
DNA metabolism	1.6	2.6	2.7	2.0
Dormancy and sporulation	0.1	0.1	0.1	0.1
Fatty acids, lipids, and isoprenoids	1.8	1.7	2.4	1.8
Iron acquisition and metabolism	0.2	0.2	0.2	0.1
Membrane transport	3.0	5.7	2.8	2.5
Metabolism of aromatic compounds	2.3	2.9	1.1	1.9
Miscellaneous	7.0	7.9	4.7	7.2
Motility and chemotaxis	1.6	0.9	2.3	1.5
Nitrogen metabolism	1.2	1.8	1.9	1.7
Nucleosides and nucleotides	3.5	3.7	3.4	3.5
Phages, prophages, transposable elements, plasmids	4.3	4.5	3.5	4.6
Phosphorus metabolism	0.1	0.4	0.2	0.9
Photosynthesis	0.5	0.5	0.7	0.5
Potassium metabolism	0.5	0.1	0.3	0.0
Protein metabolism	11.1	11.0	13.6	14.7
RNA metabolism	10.4	9.6	9.3	10.7
Regulation and cell signalling	2.7	3.3	2.5	3.5
Respiration	7.1	6.2	10.0	7.7
Secondary metabolism	2.4	1.5	0.9	0.6
Stress response	3.7	3.8	4.5	5.2
Sulphur metabolism	2.7	0.9	1.5	1.0
Virulence, disease and defence	1.7	2.3	2.6	3.0

Table 2. Per cent abundance of different functional categories in pack animals.

3.3.2. Carbohydrates

Central carbohydrate metabolism was the major functional group under carbohydrate metabolism in pack animals (**Figure 4**). The second richest functional group in carbohydrate metabolism was genes associated with carbon dioxide fixation in camel and mule and one-carbon metabolism in horse and donkey. Genes associated with one-carbon metabolism and fermentation were higher among all equidae members compared to camels. Glycoside hydrolases were seen exclusively in horses. Polysaccharide-associated genes were not seen in donkeys.

Figure 3. Per cent abundance of sub-categories in protein metabolism for pack animals. The data were compared to SEED subsystems using a maximum e-value of 1e−5, a minimum identity of 80% and a minimum alignment length of 30 measured in aa for protein.

3.3.3. Amino acids and derivatives

Amino acids and derivatives form one of the abundant functional categories in camels (7.9%) (**Figure 5**). The genes associated with aromatic amino acids and derivatives were higher in camels. Arginine, urea cycle and polyamines genes were higher in horse compared to others. In mule, genes associated for aromatic amino acids and derivatives as well as branched chain amino acids were higher compared to other sub-category genes. In donkeys, branched chain

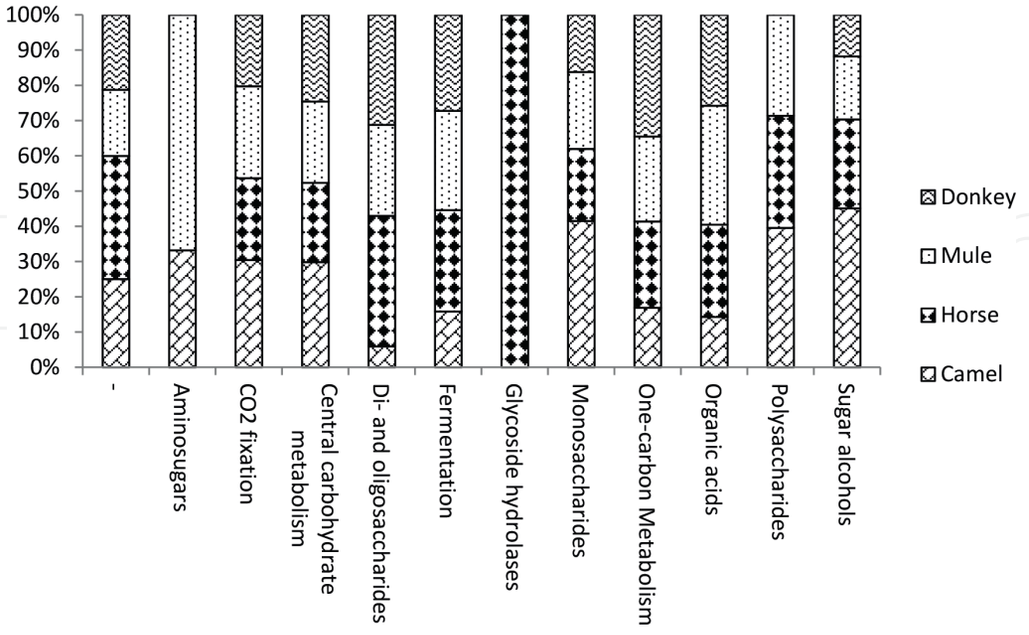

Figure 4. Per cent abundance of sub-categories in central carbohydrate metabolism for pack animals. The data were compared to SEED subsystems using a maximum e-value of 1e−5, a minimum identity of 80% and a minimum alignment length of 30 measured in aa for protein.

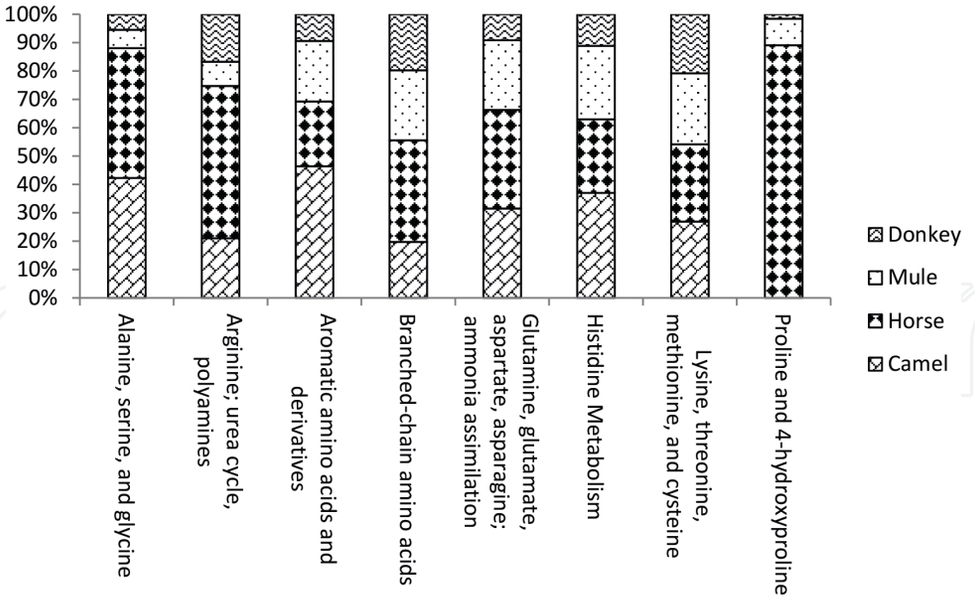

Figure 5. Per cent abundance of sub-categories in amino acids and derivatives for pack animals. The data were compared to SEED subsystems using a maximum e-value of 1e−5, a minimum identity of 80% and a minimum alignment length of 30 measured in aa for protein.

amino acids were higher. In camels, genes associated for proline and 4-hydroxyl proline metabolism were absent, lowest in mules and higher in horses.

3.3.4. Virulence, disease and defence genes

A suite of genes associated with resistance to antibiotic and toxic compounds (RATC) was highest in pack animals (**Figure 6**). The genes assigned for the virulence and antibiotic resistance

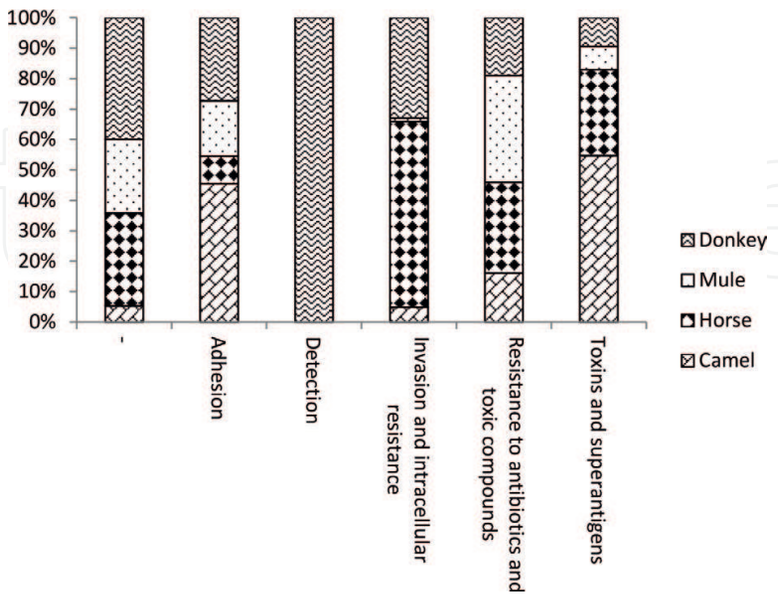

Figure 6. Per cent abundance of sub-categories in virulence, disease and defence for pack animals. The data were compared to SEED subsystems using a maximum e-value of 1e−5, a minimum identity of 80% and a minimum alignment length of 30 measured in aa for protein.

revealed abundant FemC, factor associated with methicillin resistance in all pack animals. The sub-category of detection genes was seen only in donkeys.

3.4. Comparison of microbial diversity at taxonomic and functional levels in pack animals

Comparative taxonomic and functional similarity of the pack animal faecal metagenomes was compared for generating heat maps. Hierarchical clustering of taxonomic profiles of pack animals derived from faecal metagenomes revealed that horse and donkey are closely similar (**Figures 7**). Functional similarity of samples investigated in the present study revealed that donkey and mule are closely related (**Figures 8**). The comparative metagenomic approach used in this study identified unique and/or over-abundant taxonomic and functional elements within metagenome projects.

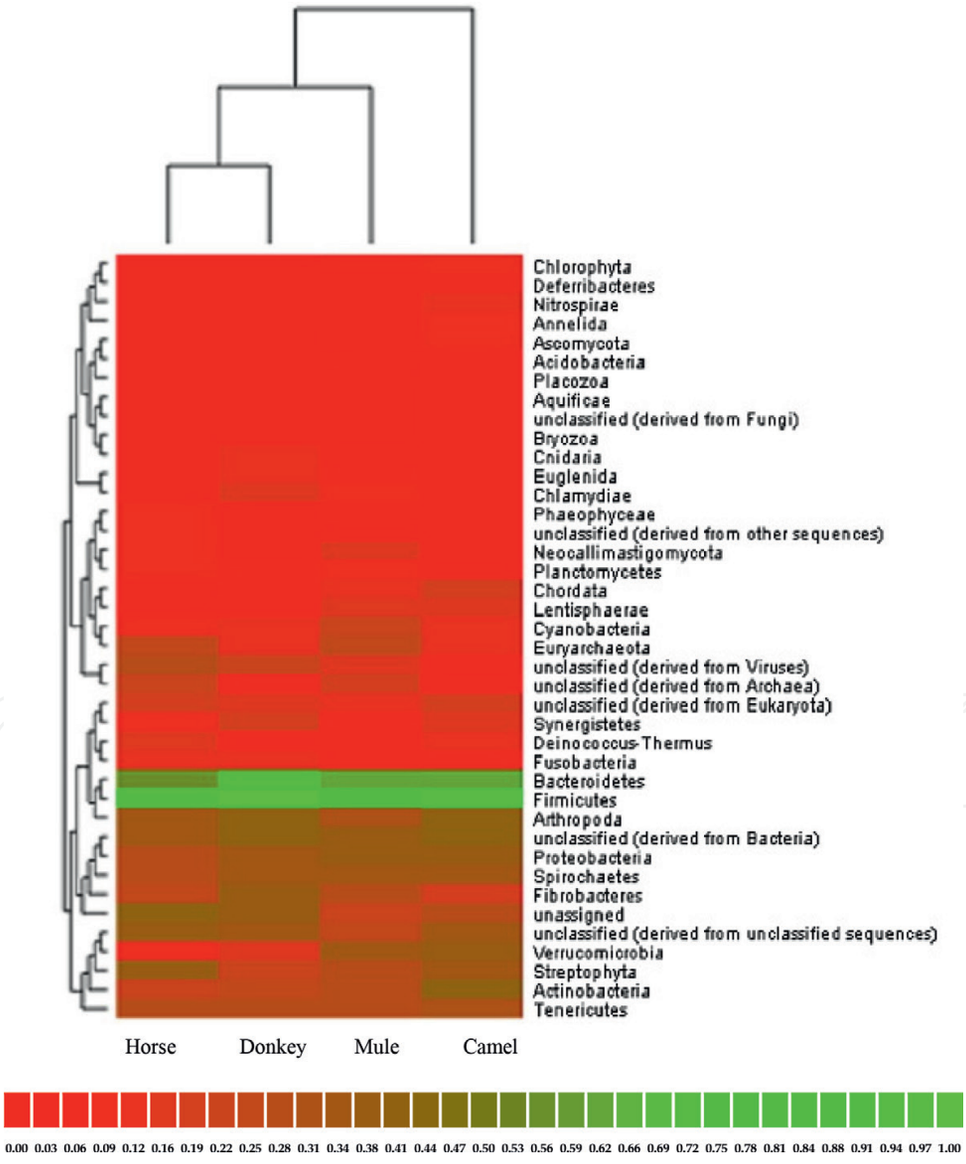

Figure 7. Heat map for pack animals microbial diversity at phylum level. The data were compared to M5RNA database using a maximum e-value of 1e−5, a minimum identity of 80% and a minimum alignment length of 50 bp.

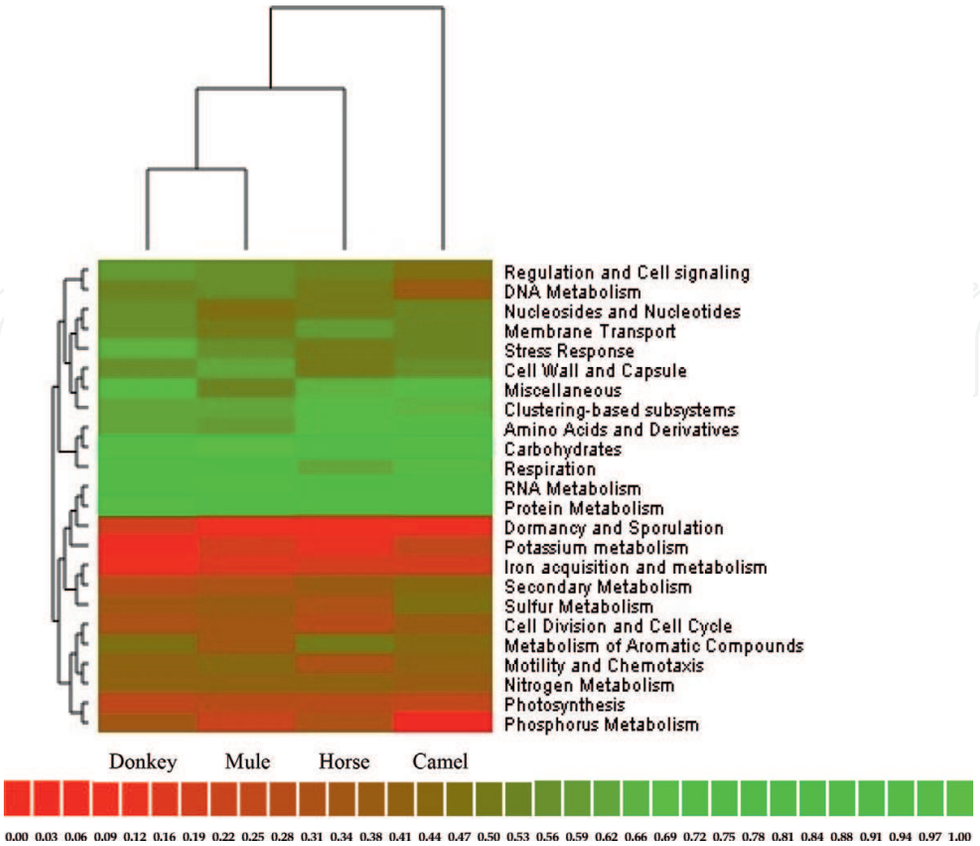

Figure 8. Heat map for pack animals microbial diversity at functional level. The data were compared to SEED subsystems using a maximum e-value of 1e−5, a minimum identity of 80% and a minimum alignment length of 30 measured in aa for protein.

Acknowledgements

The authors acknowledge the facilities provided by ICAR-NRCC, Bikaner, and Department of Biotechnology, AAU, as well as the financial assistance provided by the VTC-Rumen microbes.

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