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EXPLORING THE ROLE OF GUT BACTERIA IN DIGESTIVE SYSTEM OF COW

Alveena Akram[1a], Farzana Shahin[1b*], Iqra Asif[1c], Asif Bilal[1d], Khurram Javed Abbas[1e], Eman Younas[2]

[1]Department of Biological Sciences, Superior University Lahore, Sargodha Campus, Sargodha Pakistan

[2]Department of Biological Sciences, Superior University Lahore, Lahore Pakistan

Email: [1b]Farzana.shahin@superior.edu.pk

Email: [1d]asif.bilal.sgd@superior.edu.pk

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Corresponding Author:
Farzana Shahin, Department of Biological Sciences, Superior University Lahore, Sargodha Campus, Sargodha Pakistan
Email: Farzana.shahin@superior.edu.pk

ABSTRACT

The digestive system of cow, specifically rumen has complex and diverse ecosystem that has a significant impact on the breakdown of plant-based compounds, fermentation and absorption of nutrients. The purpose of this study was to analyze the structure, variety and functioning of rumen bacteria involved in the digestion of cow by using 16srRNA sequencing. This study provides the deep insight of bacteria involvement in digestion of cow, by identifying the composition of microbial community and their metabolic role. Genomic DNA was isolated using a fecal sample and amplified via PCR to target the 16S rRNA region of gene. After library construction and quantification sequencing was performed on the DNBSEQ platform. The raw information was filtered by eliminating poor-quality reads, adapters, sequence with low ambiguous bases and low complexity areas. 97.83% of original sequence was obtained as high-quality reads representing the excellent data integrity. Paired end reads overlapped with each other to create consensus tags for further analysis. Tags were clustered to OTU (operational taxonomic unit) with 97% similarity, producing 326 distinct OTUs in the sample. By using the Ribosomal Database project (RDP) and green-genes database taxonomic classification was performed indicated the presence of diverse microbial bacterial groups that are commonly found in ruminants. The key taxa are Oscillospiraceae, Lachnospiraceae, Bacteroidaceae, and Erysipelotrichaceae. These bacteria are recognized for their significant contributions to the production of volatile fatty acid (VFAs), fermentation of fiber, and degradation of cellulose.

INTRODUCTION

The cow, a ruminant animal vital to world agriculture, relies significantly on a distinct and complicated digestive system to convert otherwise indigestible plant matter into high-quality protein and energy. Cows, unlike monogastric animals like pigs or humans, have a four-chambered stomach consisting of the rumen, reticulum, omasum, and abomasum, with the rumen serving as the primary site for microbial fermentation. This particular adaptation allows ruminants to obtain significant nutritional benefit from cellulose-rich forages that would otherwise pass undigested through a simple digestive system. [1] Six of the over 200 ruminant species that have been recognized so far have been domesticated, with the most researched being the dairy cow. Previous research sheds light on our understanding of their intestinal processes. Foregut fermentation is a mechanism used by ruminants, primarily herbivores, to break down plant cellulose components. However, the capacity to generate enzymes that break down cellulose and other complex polysaccharides was lost by vertebrates during evolution. For the purpose of digesting these substances, ruminants depend on a symbiotic connection with microbes. The microbiota produces enzymes to break the complex compounds into simpler molecules for easy absorption by the intestine. [2]

2.2. Anatomy of Cow's Digestive System

The rumen, reticulum, omasum, and abomasum make up the quadripartite digestive system of the ruminant stomach. The reticulum directs the feed that is ingested toward the rumen. The feed used for ruminating and interacting with microorganisms is stored in the reticulumen, the collective chamber of the reticulum and rumen. Before being eaten, the feed is chewed to combine it with saliva [33]. The feed is then transferred to the next chamber, the omasum, once the feed particles have broken down into smaller compounds. The omasum functions as a filter that allows particles smaller than 2 mm to freely flow through. The digested feed then travels to the actual stomach, or abomasum. Lysozyme, a unique enzyme found in the abomasum, targets bacterial cell walls. The way that bacterial proteins and digesta are broken down in the abomasum is comparable to that of other non-ruminants. [3]

2.3. Microbial Diversity in the Rumen

Bacteria, protozoa, and fungi are among the numerous anaerobic microorganisms that coexist symbiotically with their hosts in the ruminant's gastrointestinal tract (GIT). These microorganisms receive their nourishment from the feed that ruminants consume, and microbial communities are

crucial in facilitating the host's digestion and utilization of dietary nutrients. Although ruminants are unable to secrete digestive enzymes, the rumen is one of the most important locations for feed digestion. The rumen's symbiotic microbes are solely responsible for the meal digestion process. [4] Numerous species and kinds of microbes inhabit ruminant digestive systems, and their primary function is the breakdown of nutrients, mostly cellulose and hemicelluloses. Bacteria, archaea, fungi, and protozoa make up 95% of the rumen, an anaerobic digestion chamber found in ruminants. The end products include volatile fatty acids (VFA), mostly propionate and butyrate, which are the animal's primary energy source and directly affect bodily functions, such as production rates. [5-31] The rumen microbial community is vital to life generation and maintenance because it aids in the breakdown and utilization of difficult-to-digest fiber, providing 60–85% of the amino acids that enter the small intestine and roughly 70% of the energy required by the host. [6-32] Complex structural carbohydrates are fermented by the fiber digester microbes (bacteria, protozoa, and fungi) using cellulase, hemicellulose, esterase, and pectinase. These microbes also ferment protein, non-protein nitrogen compounds, starch, and soluble carbohydrates. [7-30]

2.4. Functional Role of Gut Bacteria in Cow's Digestion

The majority of the gut microbiota is made up of bacteria, which are essential to the dairy cow's health. Through the release of several enzymes, they facilitate the fermentation and breakdown of plant polymers. Cellulose fibers are embedded in a hemicellulose matrix that makes up a plant's cell wall. A specific bacterial taxon that secretes cellulolytic enzymes is responsible for the first breakdown of this matrix. These break down cellulose into smaller oligo-disaccharides, which other organisms can subsequently react with. Fibrobacter succinogenes, Ruminococcus albus, and Ruminococcus flavefaciens are among the first-order cellulolytic bacteria. [8]

In order to support growth of cow, productivity, and host health, intestinal bacteria break down the feedstuffs and transform them into vitamins, microbial protein, and volatile fatty acids. Additionally, GIT bacteria are essential for the cattle's immune system development. [9]

Aims and objective

1. To identify the different kinds of bacteria found in the cow's digestive tract and their distinct functions in digesting.
2. To study the relationship between bacterial populations and the health of cow.

4. METHODOLOGY

4.1. Overview of the Experiment's Workflow

To perform the Polymerase Chain Reaction (PCR), two key elements are required: 16S rRNA synthesis primers and 30 ng of a verified DNA template.These are used to purify the PCR results before final labeling and library assembly. This device determines the quantity and composition of the library, and sequencing is determined based on the size of their inserts.

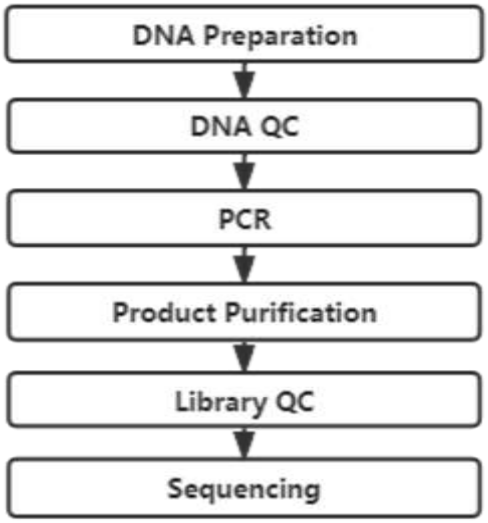

Figure 1: Overview of the experiment workflow.

4.2. Sample Collection

Sterilized equipment was used to collect samples, and samples were gathered early in the morning to minimize variability. Fecal samples were stored at -20°C in a freezer and transported on ice packs to the laboratory for further processing. Proper storage and handling ensured the preservation of sample integrity. [16]

4.3. Sample Preparation

100g of each fecal sample was weighed and placed in Eppendorf tubes, followed by the addition of buffer and sealing of the tubes. [17]

4.4. DNA Preparation

Cell Lysis: The microbial cells were lysed using a kit's lysis solution and vortexed for five seconds.

Protein Removal: After adding the inhibitor removal buffer, the mixture was vortexed to separate inhibitors from the DNA.

DNA Binding: The mixture was transferred to the QIAamp spin column, and centrifuged to check for DNA binding.

Washing: The QIAamp spin column underwent a wash step with Buffer AW2, and additional centrifugation to dry the membrane.

Elution: Elution buffer was added, followed by a one-minute centrifuge step to elute the material. [18]

4.5. DNA Quantification

DNA quantification was performed using the Qubit fluorimeter by preparing the working solution, adding DNA samples, and measuring concentration. [19]

4.6. PCR and Library Preparation

PCR Amplification of 16S rRNA Gene: Primers targeting hypervariable domains V1-V9 of the 16S rRNA gene were used for amplification. [20]

PCR Reagents: The following reagents were used for PCR:

- DNA template
- Forward and Reverse primers
- Taq polymerase
- dNTPs
- MgCl2
- PCR water
- Final volume of 20 µL

Components of PCR	Initial Concentration	Final Concentration	Volume	Volume per Reaction × n
DNA template			1 µL	1 µL x n
Forward Primer	10 µM	0.2 µM	0.4 µL	0.4 µL x n
Reverse Primer	10µM	0.2 µM	0.4 µL	0.4 µL
Taq Polymerase	5 U	1 U	0.3 µL	0.3 µL
dNTPs	10 mM	0.2 mM	0.4 µL	0.4 µL
$MgCl_2$	25 mM	1.5 mM	2 µL	2 µL

Buffer	10 x	1 x	2 µL	2 µL
PCR H_2o			13.5µL	13.5µL
Final Volume			20 µL	

PCR reactions were carried out using a Galaxy XP Thermal Cycler with the optimized PCR conditions detailed in the table.

Steps	**Sub-Cycles**	**Temp and Time**	**Cycles**
Initial Denaturation		95°C , 10 min	1
PCR Cycle	Denaturation	95°C , 1 min	40
	Annealing	60°C , 1min	
	Extension	72°C , 1 mint	
Final Extension		72°C , 10 mint	1
Hold		4^0C	1

4.7. Product Purification

Magnetic microbeads were used to purify PCR products, removing contaminants and ensuring the correct size of DNA fragments. After bead addition, tubes were placed on a magnetic stand, the supernatant was discarded, and the beads were washed with 70% ethanol. The final product was eluted and labeled. [21]

4.8. Library Quantification

The size and concentration of the library were determined using the Agilent 2100 Bio-analyzer. Electropherograms were generated to evaluate library quality and usability. [22]

4.9. Sequencing

DNA Sequencing: After DNA purification and quantification, the samples were sent to HiTech BioTech Lab Lahore for sequencing of the 16S rRNA gene (V3-V4 region).

4.10. Data Filtering

Data was filtered to exclude low-quality reads, adapter-contaminated sequences, reads containing 'N' bases, and low complexity reads. Clean reads were matched to their corresponding samples using internal scripts. [23]

4.11. Tags Connection

Overlapping paired-end reads were merged using FLASH software with a stretch of at least 15 bp overlap and an inconsistency ratio of ≤ 0.1. [24]

4.12. OTU Clustering

Sequences were classified into Operational Taxonomic Units (OTUs) based on 97% similarity using USEARCH.UCHIME was used to filter out chimeric sequences. USEARCH was used for mapping and calculating OTU abundance.

4.13. Taxonomy Annotation

OTU sequences were aligned against taxonomic databases (e.g., Silva, Greengenes, UNITE) to annotate the taxonomy at various levels. [25]

4.14. Core-Pan OTU Plot

This plot visualizes the typical and unique OTUs across samples or groups, allowing for comparison between five or fewer groups. [26]

4.15. OTU Rank Curve

Species Diversity and Abundance: The OTU rank curve demonstrated species diversity and abundance patterns across samples.

4.16. Functional Prediction

Functional annotations were predicted using PICRUSt2, linking OTUs to gene families and metabolic pathways. The functional abundance for KEGG pathways and COGs (Cluster of Orthologous Groups) was estimated. [27-30]

3.17 Correlation Analysis and Model Prediction

Network Analysis: Cytoscape software was used to analyze species interactions, focusing on metabolic functions and species abundance correlations. [28-29]

5 RESULTS

High-throughput sequencing methods with bioinformatics tools examined cattle rumen microorganisms according to the research. Quality filtration occurred before FLASH software performed tag synchronization on the data. USEARCH conducted 97% similarity-based cluster analysis of OTUs before RDP Classifier performed the annotation. The analysis collected data about OTUs and diversity measurements together with species identity assessments and examination of abundance metrics. The PICRUSt2 software generated function predictions by

processing pathway data obtained from KEGG and COG and MetaCyc platforms. The network analysis was performed using Cytoscape and R (v3.4.1).

5.1. Data Filtering

The following filters are used to process raw data to produce clean, high-quality reads:

- Reads will be shortened if their average Phred quality values during a 25 bp sliding window are less than 20.
- Eliminate reads that contain adapter sequences. The reads and adapter overlap by at least fifteen bases, allowing up to three mismatched bases.
- Eliminate reads with unknown bases (N base).
- Eliminate simple sequence reads with 10 successive same bases.
- Eliminate reads that are 75% of their initial length after trimming.
- Clean readings were allocated to matching samples by using in-house scripts to align against barcode sequences having zero bases mismatch in order to guarantee that barcode sequences were eliminated from pooling libraries.

The data filtering process relied on software applications including iTools Fqtools fqcheck (v.0.25), together with cutadapt (v.2.6), and read (v1.0).

Table 5.1 Data Filtering

Sample Name	ReadsLength (bp)	RawData (Mbp)	NBase (%)	PolyBase (%)	LowQuality (%)	Clean Data (Mbp)	Data Utilization Ratio (%)
A1	300.300	38.331	0.898	0.001	0.032	37.50	97.83

5.2. Tags Connection

When overlap between paired-end reads occurs, FLASH (Fast Length Adjustment of Short Reads) generates a conserved sequence. Details are provided as follows:

Least overlap stretch: 15 bp.≤ 0.1 is the overlapping region's inconsistency ratio.

Table 5.2 Tags Connection

Sample Name	Total Pairs Read Number	Connect Tag Number	Connect Ratio (%)	Average Length And SD

A1	68366	66144	96.75	425/8

5.3. OTU Clustering

OTUs (Operational Taxonomic Units) in the phylogenetic research serve as single indicators for examining taxa across seven taxonomy levels. The research requires 97% sequence similarity to group OTUs and measure bacterial counts at all sample levels.

5.3.1. OTU Cluster

Using USEARCH software, tags are grouped according to OTU, specifics are provided below:

- UCHIME (v4.2.40) filters the chimeras.
- The UPARSE program generates OTU clusters by setting a 97% threshold and produces distinct representative sequences for each OTU cluster. De novo chimera detection analysis was conducted on the 18S rDNA sequences with a threshold of 0.1.
- OTU chimeras were filtered and tested for 16S rDNA and ITS sequences, which were mapped to the Gold database and UNITE for identification.
- USEARCH GLOBAL is used to map all tags to OTU indicative sequences in order to calculate the OTU abundance table.

5.3.2. OTU Statistics

Table 5.3.2 OTU Statistics

Sample Name	Tag Number	OTU Number
A1	58756	326

5.3.3. OTU Taxonomy Annotation

RDP Classifier (v2.2) program aligns OTU representative sequences against the taxonomic annotation database, with the program establishing 0.6 as its default threshold for sequence identity.

Database:

18S Fungus: SilvaV138 2019-12-16.

16S (Bacterial and archaeal groups): Greengene V201305, RDP[30].

ITS Fungus: UNITE Version 8.2 2020-02-20.

The following filters were applied to annotation results:

- Eliminated OTUs that were not tagged.
- Eliminated taxonomies that did not align with the preliminary research of the project; for example, if 16S bacteria samples were provided, any OTU annotated as Archaea were eliminated, leaving the remaining OTUs for further analysis.

Table 5.3.3 OTU Taxonomy Annotation

OTUId	Abundance	Taxonomy
Otu308	6	**Bacteria; Bacillota; Clostridia;Eubacteriales;Oscillospiraceae;Pseudoflavonifractor;Pseudoflavonifractor_gallinarum**
Otu313	2	**Bacteria;Bacillota;Clostridia;Eubacteriales;Oscillospiraceae**
Otu129	3	**Bacteria;Mycoplasmatota;Mollicutes;Acholeplasmatales;Acholeplasmataceae;Acholeplasma**
Otu128	8	**Bacteria;Bacillota;Erysipelotrichia;Erysipelotrichales; Erysipelotrichaceae**
Otu127	8	**Bacteria;Bacillota;Clostridia;Eubacteriales;Lachnospiraceae**
Otu126	12	**Bacteria;CandidatusSaccharibacteria;Unclassified;Unclassified;Unclassified;Saccharibacteria**
Otu125	12	**Bacteria;Actinomycetota;Actinobacteria;Micrococcales;Microbacteriaceae;Microbacterium**
Otu124	13	**Bacteria;CandidatusSaccharibacteria;Unclassified;Unclassified;Unclassified;Saccharibacteria**
Otu123	9	**Bacteria;Bacteroidota;Bacteroidia;Bacteroidales;Porphyromonadaceae;Parabacteroides;Parabacteroides_goldsteinii**
Otu12	14	**Bacteria;Bacteroidota;Bacteroidia;Bacteroidales;Bact**

2		**eroidaceae;Bacteroides;Bacteroides_stercoris**
Otu121	5	**Bacteria;Bacillota;Clostridia;Eubacteriales;Oscillospiraceae**
Otu120	4	**Bacteria;Bacillota;Clostridia;Eubacteriales;Oscillospiraceae**
Otu61	28	**Bacteria;Bacillota;Clostridia;Eubacteriales;Oscillospiraceae;Lawsonibacter**
Otu60	20	**Bacteria;Actinomycetota;Coriobacteriia;Eggerthellales;Eggerthellaceae;Adlercreutzia**
Otu63	13	**Bacteria;Bacillota;Clostridia;Eubacteriales;Lachnospiraceae**
Otu62	25	**Bacteria;Bacillota;Clostridia;Eubacteriales**
Otu65	76	**Bacteria;Bacillota;Clostridia;Eubacteriales;Oscillospiraceae;Vescimonas**
Otu64	22	**Bacteria;Bacteroidota;Bacteroidia;Bacteroidales;Muribaculaceae;Muribaculum**
Otu67	13	**Bacteria;Bacillota;Clostridia;Eubacteriales;Lachnospiraceae**
Otu66	**10**	**Bacteria;Bacillota;Clostridia;Eubacteriales;Oscillospiraceae;Hominimerdicola;Hominimerdicola_aceti**

5.3.4. Core-Pan OTU Plot

Observations of both core shared and distinctive OTUs appear in the chart known as the Core-Pan OTU. The Core-Pan OTU plot functions similarly to a Venn diagram since it handles the analysis of up to five groups or samples. Software: R(v3.1.1) constituted the instrument.

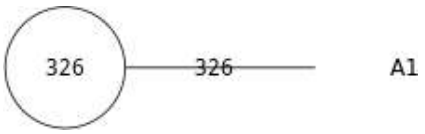

Figure 5.3.4 Core-Pan OTU Plot

5.3.5. OTU Rank Curve

The OTU rank curve displays the species diversity through both richness and evenness measurements between various samples or groups. Researchers determined the relative abundance of each OTU within every sample while maintaining a descending order list of species. The OTU relative abundance appears on the Y-axis, and the OTU rank runs along the X-axis. The OTU rank curve was built using software R version 3.1.1.

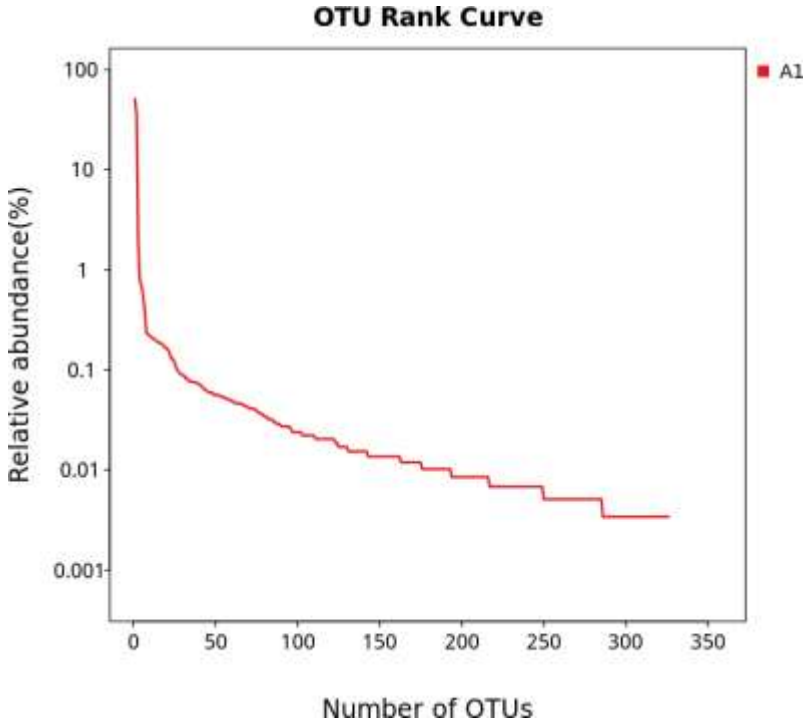

Figure 5.3.5 OTU Rank Curve

5.4. Species Composition and Abundance

The bacterial composition analysis of OTU representative sequences depends on the RDP classifier Bayesian method. The conclusion of annotation leads to the generation of species abundance calculations at Phylum, Class, Order, Family, Genus, and Species levels.

5.4.1. Barplot Showing Species Abundance

The species abundance barplot displays the relative frequencies of species that occur within each particular group of samples. Any species with relative abundance lower than 0.5 will be grouped as “others”. The barplot was created using software R.

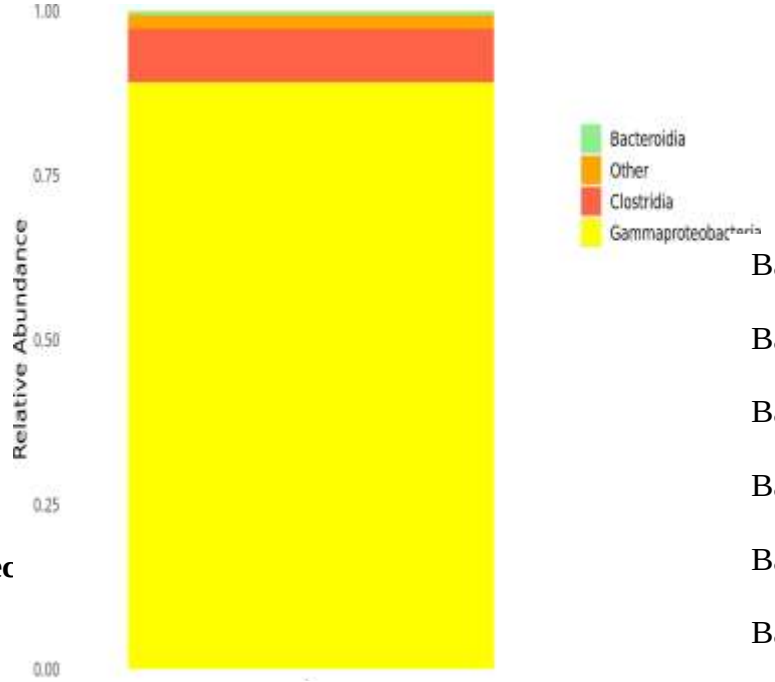

Figure 5.4.1 Spec

Barplot.Group.Cla

Barplot.Sample.Cl

Barplot.Sample.Cl

Barplot.Group.Fa

Barplot.Sample.Fa

Barplot.Sample.Fa

A species with relative abundance below 0.5% will receive the classification of "others."

5.4.2. Species GraPhlan Map

This software program creates excellent phylogenetic tree and circular taxonomy presentations. The species GraPhlan Map was created using GraPhlAn software.

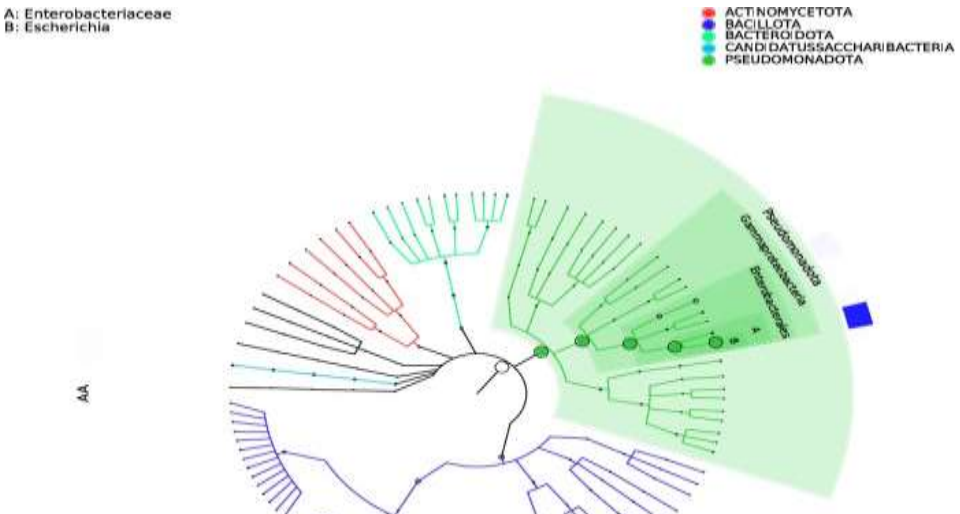

Figure 5.4.2 Species GraPhlan Map

The five circles arranged from inside to outside represent phylum then class followed by order then family before concluding with genus. The phylum-level genera display different node colors throughout the tree diagram while the node size corresponds to the abundance of each genus found in the stomach content.

5.4.3. Species Phylogenetic Analysis

A phylogenetic tree is a representation of the evolutionary lineage among species. Phylogenetic trees are theoretical models, with branching structures illustrating how species or other groupings split off from common ancestors. The analysis was conducted using FastTree software and the trees were displayed using R software version 3.1.1.

Figure 5.4.2 Species phylogenetic analysis.

Phylogenetic tree. The pattern of branching in a phylogenetic tree reflects how species or other groups evolved from a series of common ancestors.

5.5. Divers

Six indexes are employed to measure alpha diversity, including observed species index, Chao index, ACE index, Shannon index, Simpson index, and Good-coverage index. The first four values correlate directly with species diversity, while the Simpson index reflects an opposing relationship.

A higher good-coverage score indicates fewer unknown species within the examined samples. The observed species index, Chao index, and ACE index reveal the variety of microbes, and rarefaction curves of these indices help determine if sequencing data encompasses all detectable species.

5.5.1. Statistical Table of Alpha Diversity

Table 5.5.1 Statistical table of alpha diversity

Sample Name	Sobs	Chao	Ace	Shannon	Simpson	Coveage
A1	326.000000	326.000000	326.000000	1.593504	0.386635	1.000000

Alpha diversity's mean and standard deviation for every group. There are substantial variations in species diversity across groups The significance of differences between alpha diversity of at least two groups becomes apparent when the p value falls below 0.05.

5.6. Function Prediction

PICRUSt predicts the microbial functional annotation, offering a greater understanding of microbial diversity. It predicts gene families and enzyme classifications through sequencing profiles. The PICRUSt2 primary pipeline consists of determining optimal OTU placement in a reference phylogenetic tree, performing hidden-state prediction, and deducing gene and pathway abundances in samples.

5.6.1. KEGG Function Prediction

PICRUSt2 was used to determine the bacterial community's projected KEGG function abundance. The function utilizes the KEGG database to obtain three levels of metabolic pathway information, and software R(v3.4.10) facilitated KEGG function prediction.

5.6.2. COG Function Prediction

COG function abundance predictions were conducted using PICRUSt2. The two levels of the COG database were analyzed with R(v3.4.10) and PICRUSt2 v2.3.0-b.

5.6.3. Histogram of Predicted COG Profile

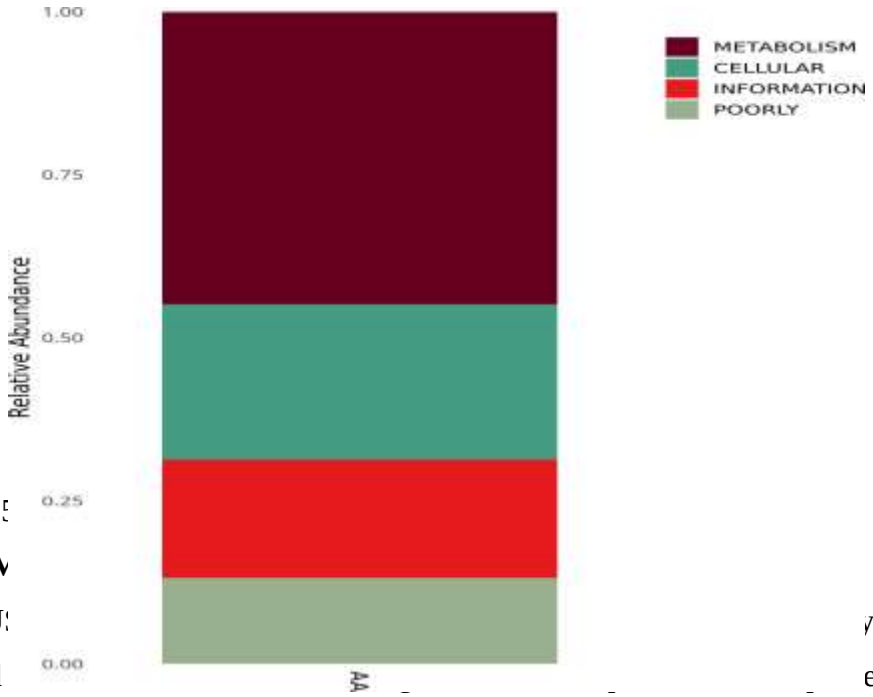

Figure 5

5.6.4 M

PICRU s based on metabolites created essed using PICRUSt2 v2.3.0-b and R (v3.4.10).

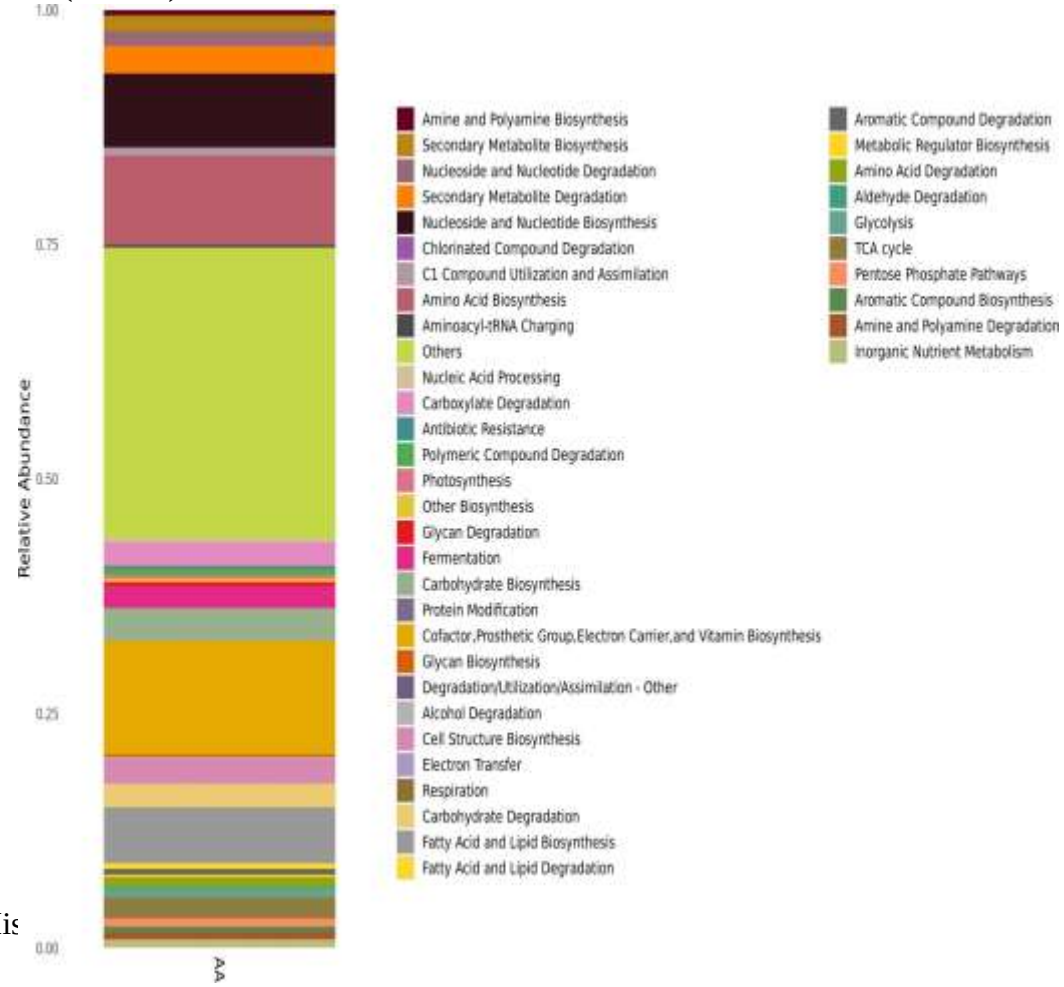

Figure 9 His

5.7. Correlation Analysis and Model Prediction

5.7.1. Network Analysis

Cytoscape was used to display relationships between species/metabolic functions and samples, revealing significant information networks based on species abundance and interactions. The network analysis was performed using Cytoscape and R (v3.4.1).

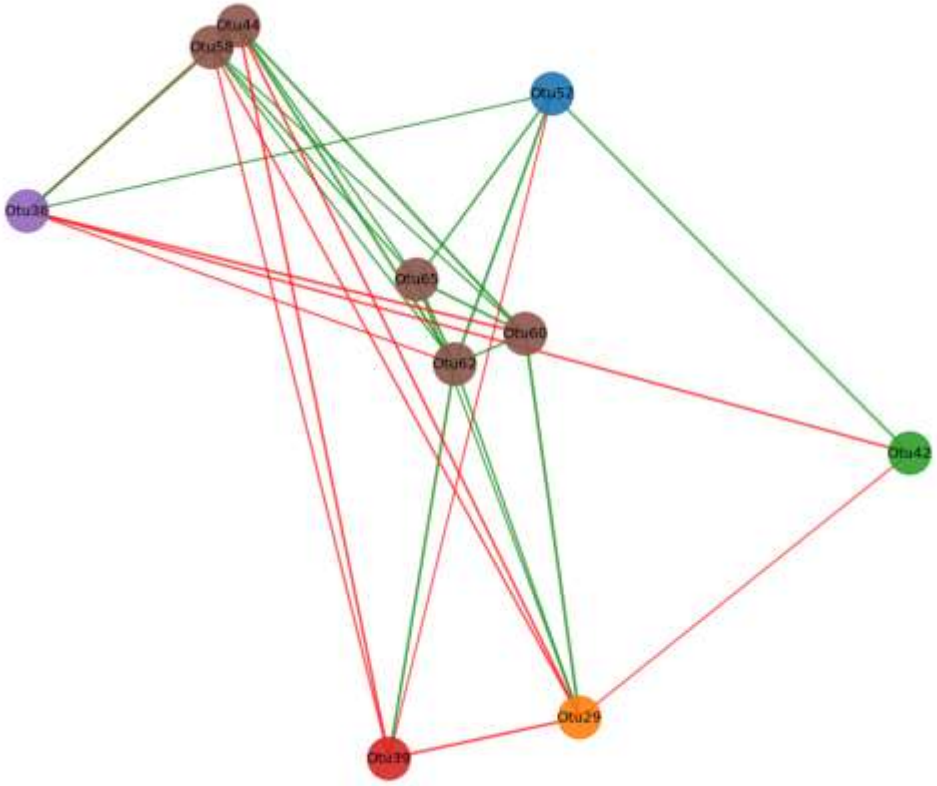

Figure 10 Network Analysis

Discussion

The rumen function depends heavily on bacterial digestion activities. The rumen constitutes the major stomach chamber in cows where different microbes collaborate to process plant fiber while facilitating nutrient acquisition. Rumen bacterial structure together with their digestive functions was assessed by researchers utilizing 16S rRNA gene sequencing and bioinformatics methods. Scientific evidence shows different rumen bacterial species present in cows and their specific operation in their digestive process. The sequencing data revealed 326 distinctive OTUs representing a wide range of bacterial species in the community. Researchers studied bovine rumen

microbiome biodiversity effects on digestive efficiency in previous investigations. High-throughput sequencing methods revealed multiple bacterial microbes for both taxonomic groups and species while providing comprehensive information about four bacterial families known as Oscillospiraceae, Lachnospiraceae, Bacteroidaceae, and Erysipelotrichaceae. Volatile fatty acids produced from complex carbohydrate fermentation act as main energy sources for cows and their production is regulated by particular bacterial families. Firmicutes and Bacteroidetes were the dominant phyla, which was accordant with published literatures about ruminant gut microbiomes (Jami and Mizrahi, 2012). Firmicutes, particularly those classified within the families Ruminococcaceae and Lachnospiraceae, also are known as major fiber degraders with preference for degrading complex polysaccharides, such as cellulose and hemicellulose. Bacteroidetes phylum's Prevotella species breaks down non-cellulosic polysaccharides along with protein components of the substrates by enzymatic degradation which provides the cow with nutrients from bulky feeds. At the same time, Proteobacteria remain at low abundance in the lower GI tract. This phylum contains numerous members that carry out nitrogen metabolic functions while they may participate in maintaining redox balance within the gut.

Conclusion

The cow's digestive system hosts a large, diverse community of bacterial organisms that thrive in the rumen, enabling the breakdown of plant materials like cellulose and hemicellulose. These microbes produce volatile fatty acids and microbial proteins essential for energy, growth, and milk production. Dietary intake significantly influences bacterial composition, and balanced gut flora is vital for preventing acidosis and maintaining digestion. Gut bacteria supply nutritional resources, protect against diseases, and fulfill protein needs. Managing microbiota through diet and probiotics improves cow health and productivity. Understanding the cow-microbe relationship offers essential value for sustainable cattle husbandry and agricultural development.

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