

16s rRNA Amplicon Sequencing Approach for Community and Predictive Functional Diversity of Therapeutically Valuable Formulation of Cow-derivatives

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ABSTRACT

Panchagavya is the blend of five ingredients obtained from cows and used in traditional Hindu rituals. Panchagavya preparation is a microbial mediated process that possibly involves microbial succession. The present study was conducted to decode the microbial community that exists in the preparation of three hours of old Panchagavya. DNA was isolated from Panchagavya using HiPurA™ Stool DNA Purification Kit followed by quality checking by Agarose electrophoresis and Qubit fluorometer. The V3 and V4 region of 16s rRNA based amplicon sequencing was performed using the Illumine MiSeq platform. Taxonomic profile encoded by using One Codex, kraken and MG-RAST. Functional traits detected through the abundance of specific genes using Tax4Fun. Taxonomic result suggests the total 2000 species were identified. The most abundant was *Streptomyces griseocarneus* (2.65%) followed by *Clostridiales bacterium* (2.26%), *Bacteroidales bacterium* (1.38%), and *Verrucomicrobia bacterium* (1.13%). Community based analysis revealed the microbial diversity and presence of anaerobic, unclassified, and uncultivable microbes in metagenomes, which may be associated with the pharmacological properties of Panchagavya. Functional analysis predicts around 351 metabolic pathways for metabolism of carbohydrates, synthesis of secondary metabolites and degradation of xenobiotic compounds. The detection of various secondary metabolites genes associated with pharmacological molecules correlated with its traditional clinical applications. The present study revealed the advantages of cultivation approach for exploring untapped and unique bacterial diversity, and also utilities for various biotechnological and environmental applications.

KEY WORDS: AMPLICON SEQUENCING, COMMUNITY METAGENOMICS, COW, FUNCTIONAL METAGENOMICS, PANCHAGAVYA.

INTRODUCTION

Panchagavya is an organic product derived from five products of the cow. The three are direct constituents i.e. dung, urine and milk and the two derived products are curd and ghee. According to the old Ayurveda literature, the five individual constituents of Panchagavya possess medicinal properties and can be used singly or in combination with some other

herbs. This kind of treatment is called Panchgavya therapy or cowpathy (Muthukapalli et al., 2022).

The potential applications of Panchagavya are as antimicrobials, immune boosters, antidiabetics, anticancer, anticonvulsant, aphrodisiac, blood purifiers, and anti-HIV agents. Panchagavya is also used in Ayurveda for the treatment of several disorders such as hyperlipidemia, leucoderma, arthritis, acidity, renal disorders, asthma, dietary disorders, gastrointestinal disorders and asthma as an antistressor. It also acts as a general tonic or immunomodulator to enhance the immunity (Dhama et al., 2022, Athavale et al., 2012 & Kuldeep et al., 2013).

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Panchagavya is essential in organic agriculture for disease control, plant growth, flowering, germination, and canopy development. It also plays a significant part in compost decomposition, improves soil nutrient status by promoting better mineralization, and aids in the creation of beneficial microbes in the root rhizosphere, which can be helpful in the supply of beneficial macro and micro nutrients to plants (Kumar et al., 2022).

Panchagavya contains several macronutrients such as nitrogen, phosphorus, potassium and micronutrients like Zn, Fe, Cu, and Mn which are required for the normal growth and development of plants. In the Indian subcontinent, the application of the Panchagavya is well-known and used in an agricultural sector to protect the crops and growth promotion of the crops (Gugalia et al., 2021, Kumaravelu et al., 2009, Dhama et al., 2005, Chauha, R.S., 2002, Krupanidhi et al., 2008, Matthews and Jenks, 2013, Kumar et al., 2022).

By culture dependent study few bacterial species isolated from the Panchagavya were *Lactobacillus*, *Saccharomyces*, *Streptomyces*, and *Rhodopseudomonas* (Leo et al., 2013), *Azospirillum*, *Azotobacter*, *phosphobacteria* (Dhama et al., 2013), *Pseudomonas*, *Azotobacter*, *Actinomyces*, *Rhizobium* (Ram et al., 2013), *Acetobacter*, *Bacillus*, *Micrococcus*, *Leuconostoc*, *Enterococcus*, *Microbacterium*, *Pseudoxanthomonas*, *Corynebacterium*, *Escherichia*, *Paenibacillus*, *Shigella*, *Rhodobacter*, *Lactococcus*, etc. (Anandham et al., 2015). Nowadays, the Prophylactic potential of a Panchagavya formulation was tested against certain pathogenic bacteria (Patel et al., 2018). So, based on such diverse properties and the presence of different bacterial species; there is a need to decode the microbiome of the Panchagavya formulation. To the best of our knowledge; at present, there is not a single study of the metagenomics analysis of Panchagavya. This is the first microbial profiling of the Panchagavya by culture independent methods using a metagenomics approach.

MATERIAL AND METHODS

Panchagavya was prepared at Madhuvan Dairy Farm, Haldarva, Bharuch, India. Fresh cow dung, urine, milk, curd, and ghee were mixed in equal proportion thoroughly in a sterile glass beaker. This mixture was allowed to stand for three hours and subjected to filtration through a muslin cloth aseptically.

DNA was isolated from the filtrate using HiPurA™ Stool DNA Purification Kit (MB544) according to the manufacturer's instruction. DNA concentration was measured using the Qubit Fluorometer. The sequencing library generated from V3 and V4 amplicons from the sample were sequenced using an Illumina MiSeq sequencing platform. Diversity and abundance were analysed using available standard bioinformatics software. The taxonomic assignment of unassembled metagenomic sequences was performed using FASTQC followed by Pear, One Codex

(Minot et al., 2015) and predictive functional metagenomics performed by SILVAngs, MicrobiomeAnalyst (Dhariwal et al., 2017), Tax4fun (Abhauer et al., 2015), KO and KEGG mapper (Kanehisa & Sato 2020).

Figure 1: Krona based chart at family level. (Core of chart indicated phyla and peripheral edge indicates the family's abundance in percentage)

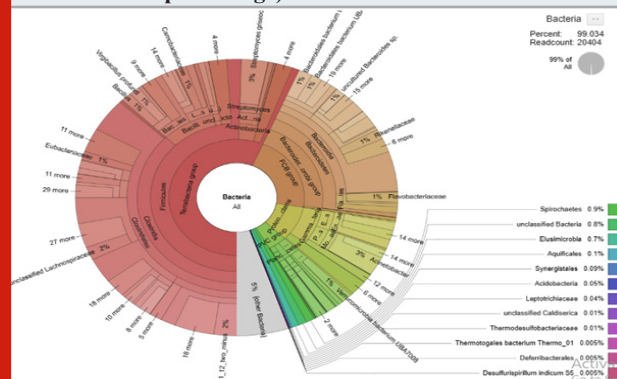
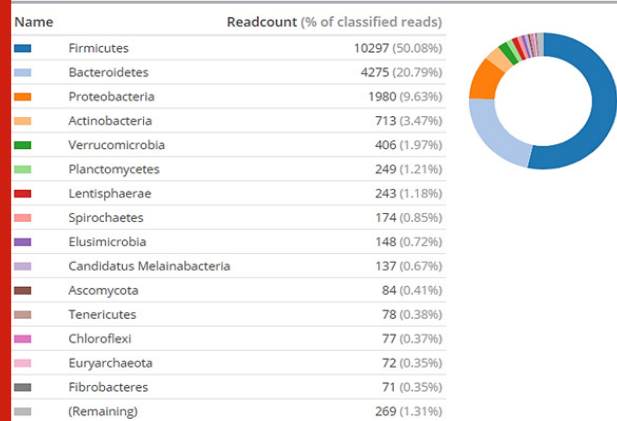


Figure 2: Community at Phyla level based on the percentage of reads in metagenome sample (Pie chart includes the most 15 dominant phyla presented in clockwise direction)



RESULTS

The present study is based on the 16s rRNA amplicon NGS metagenomic analysis of Panchagavya. A total number of 27301 bacterial 16S V3-V4 high-quality sequences with an average read length of 71 to 490, were obtained. Profiling the taxonomic composition of the community also can be accomplished by the analysis of the distribution of k-mers (e.g., using Kraken or One Codex). Metagenomic analysis using One Codex was performed by uploading the 2D FASTQ data to the One Codex platform at <https://app.onecodex.com>. This cloud-based k-mer method was selected, because it is reportedly more accurate than either the MG-RAST or the Kraken tools and it provides for community access to the data and analytical results. Sequence clustering resulted in the identification of 2000 different bacterial species, 87 diverse microbial phyla and 510 families together with an unclassified category were depicted in the metagenome (Figure 1).

Firmicutes (50.08%) and Bacteroidetes (20.79%) were abundant along with Proteobacteria (9.63%), Actinobacteria (3.47%), Verrucomicrobia (1.97%) and Planctomycetes (1.21%) (Figure 2).

Total of 516 families was detected (figure 1) along with following *Ruminococcaceae* (8.46%), *Lachnospiraceae* (7.34%), *Bacteroidaceae* (3.57%), *Enterobacteriaceae* (3.27%) and *Moraxellaceae* (3.12%) were major dominant (Figure 3).

Figure 3: Microbial communities at species level based on the percentage of reads in metagenome sample (Pie chart includes the most 15 dominant species presented in clockwise direction)

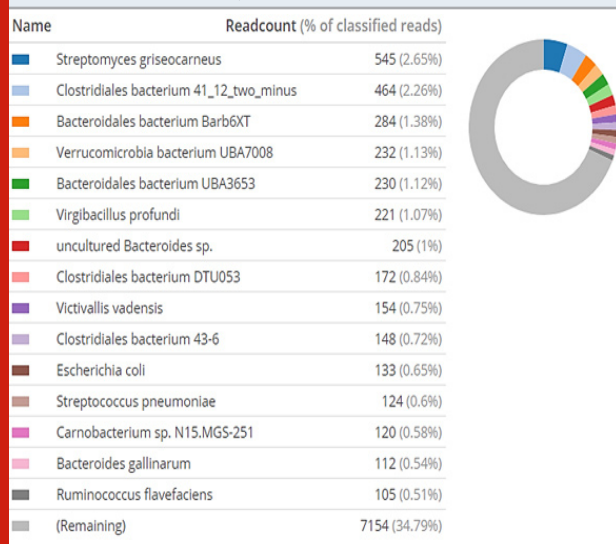
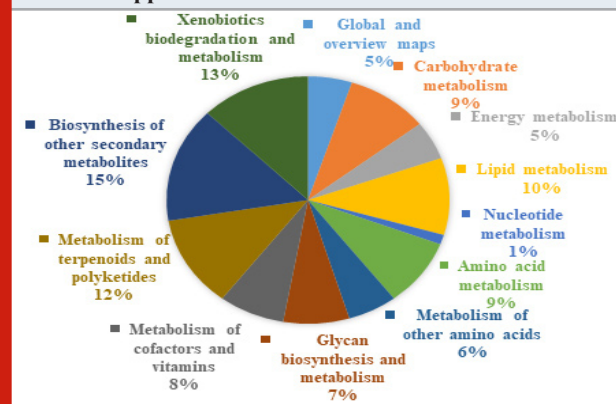


Figure 4: Secondary metabolites pathways analysis using KEGG Mapper



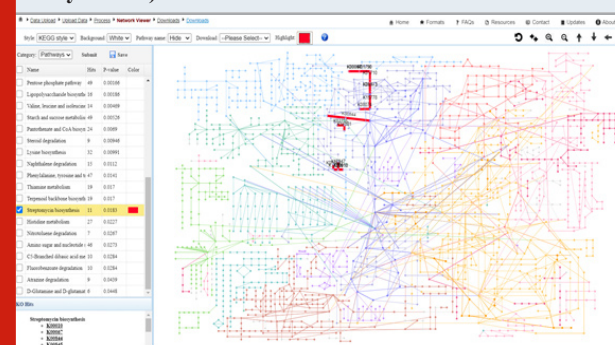
Based on the microbiome analyser, community analysis data was subjected to the functional analysis to decode the functional potential of the microbiome. In the present study pathways modules for the metabolism of cofactor and vitamins, biosynthesis of secondary metabolites, xenobiotic degradation, drug resistance, lipid metabolism, and energy metabolism were detected which correlates its pharmaceutical and agricultural application (Figure

4). Furthermore, we have analysed the 10 categories of Reconstruction Pathway (Table 1). The highest number of the pathway was found in Environmental information processing. To confirm the pharmacological and medicinal attributes of the Panchagavya, we have further analysed the Biosynthesis of other secondary metabolites pathways and were find out the 12 important biosynthetic pathways which give the antimicrobial substances. Furthermore the biosynthesis of Streptomycin was highlighted in the global metabolic network (Figure 5).

Table 1. List of reconstruction pathway found in the metagenome sample (number of pathways indicates total different pathways operated in microbiota of sample under each categories)

Sr. No.	Reconstruction Pathway	No. of Pathway
1	Carbohydrate metabolism	41
2	Energy metabolism	62
3	Lipid metabolism	32
4	Nucleotide metabolism	08
5	Amino acid metabolism	53
6	Glycan metabolism	24
7	Metabolism of cofactors and vitamins	31
8	Biosynthesis of terpenoids and polyketides	45
9	Xenobiotics biodegradation	21
10	Biosynthesis of other secondary metabolites	28

Figure 5: A depiction showing functional enrichment analysis and visualization within the global metabolic network. (Highlighted red lines shows the Streptomycin biosynthesis)



DISCUSSION

Panchagavya plays major role in crop production, especially in organic agriculture, maintaining genetic biodiversity, helps as a growth promoter, root growth enhancer, increasing water holding capacity, increase photosynthetic activity in

plants, human diseases and improving the body's immunity, the metabolic profiles (hormones, proteins, nutrients, etc.) and microbial profiles have not been explored completely (Somasundaram et al., 2007; Kumar et al., 2022).

Moreover, the profile and dynamics of the entire microbial community associated with the Panchagavya are unavailable due to limitations in culturing several genera/species using conventional microbiological techniques. Not many efforts have been made to explore the complete biology involved in agriculture, indicating its potential for increasing crop output by the Panchagavya. Only by combining different omics approaches can the fundamental aspects of a complex system be understood (Segate et al., 2013).

Therefore, we have used different omics approaches to decode the microbial community (metagenomics) and metabolite profile (metabolomics) in the Panchagavya. These approaches can reveal the microbial composition and their abundance, the functional annotation of genes and important protein compounds, hormones, and so forth, in the Panchagavya formulation. Several bacteria that used as starter cultures for dairy products were found to be predominant in Panchagavya. Among them *Lactococcus fermenticola*, *Lactococcus fujiensis* JCM 16395, and *Lactococcus fujiensis*, is widely used as starter cultures for various cheeses (Lahtinen et al., 2011). Our findings are in contrast to a recent report that the use of *Bacillus cereus*, *Bacillus subtilis*, *Lactobacillus camellia*, *Lactobacillus ozone*, and *Paenibacillus* as a probiotic dietary supplements is expanding rapidly with increasing number of studies demonstrating immune stimulation, antimicrobial activities and competitive exclusion (Kamilya et al., 2022; Cutting, 2011; Sorokulova et al., 2008).

CONCLUSION

Using metagenomic sequencing, the Panchagavya's related microbiota can be profiled. An extensive range of bacterial taxa from the domain bacteria were found in the Panchagavya, according to the research. In Panchagavya metagenomes, the Firmicutes, Bacteroidetes, and Proteobacteria phyla are widely present. Its health benefits are suggested by the presence of a few probiotic species and antibiotic-producing species. The research sheds light on the existence of several bacterial species, whose immune-boosting characteristics and therapeutic potential may be the basis for the Panchagavya's health-tonic properties. According to pathway research, the secondary metabolites that the bacteria create may be the cause of the Panchagavya's antibacterial abilities. The Panchagavya is a low-cost instrument for enhancing gut immunity and has a positive impact on health because the enormous microbial variety and the detection of rare species and uncultivable species play a crucial role in maintaining an intestinal barrier and metabolising nutrients.

Data availability statement: Metagenome sequences data are deposited in EBI-Metagenomics under the accession

number PRJEB31987.

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Conflict of Interest: The authors declare that they have no conflict of interest.

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