METAGENOMIC ANALYSIS OF PANCHAGAVYA

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CERTIFICATE

This is to certify that the thesis entitled “METAGENOMIC ANALYSIS OF PANCHAGAVYA” submitted by Mr. BALASAHEB GUNWANTRAO BIRADAR, for the degree of MASTER OF SCIENCE (AGRICULTURE) in MOLECULAR BIOLOGY AND BIOTECHNOLOGY to the University of Agricultural Sciences, Dharwad is a record of research work carried out by him during the period of his study in this university, under my guidance and supervision, and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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JULY, 2015

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"Gratitude takes three forms, a feeling from the heart, an expression in words and a giving in return….."

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LIST OF ABBREVIATIONS

bp	Base pair
cm	Centimeter
EDTA	Ethyl Diamine Tetra Acetic Acid
DGGE	Denaturing Gradient Gel Electrophoresis
rDNA	Ribosomal-Deoxyribose Nucleic Acid
rRNA	Ribosomal Ribose Nucleic Acid
g	Gram
hr	Hour
Kb	Kilobase
M	Molar
ml	Millilitre
mm	Millimetre
mM	Millimolar
ng	Nanogram
N	Normality
nM	Nanomolar
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
pmol	Picomole
ppm	Parts per million
rpm	Rotations per minute
SDS	Sodium Dodecyl Sulphate
TAE	Tris Acetic Acid
UV	Ultraviolet
µg	Microgram
µl	Microlitre
%	Per cent
°C	Degree Celsius
LC-MS	Liquid Chromatography Mass Spectrometry
LC-MS-ELSD	Liquid Chromatography Mass Mpectrometry- Evaporative Light Scattering Detector

GC-MS	Gas Chromatography Mass Spectrometry
HPLC-UV-VIS	High Pressure Liquid Chromatography- equipped with Ultra Violet and Visible Light Detector
HPLC-ELSD	High Pressure Liquid Chromatography-Evaporative Light Scattering Detector
HPLC-PDA	High Pressure Liquid Chromatography- Photodiode Array Detectors
HPCCC	High Performance Counter- Current Chromatography
CZE	Capillary Zone Electrophoresis
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
HR-MS	High Resolution Mass Spectrometry

1. INTRODUCTION

Intensive agriculture involving the use of chemical fertilizers in large amount has, no doubt, resulted in manifold increase in the productivity of farm commodities but, the adverse effects of these chemicals are clearly visible on soil structure, micro-flora, and quality of water, food and fodder. Therefore, the current global scenario firmly emphasizes the need to adopt eco-friendly agricultural practices for sustainable food production. Organic farming based on holistic, ecologically balanced agricultural principles involving soil fertility, crop rotation and natural pest control would serve the dual purpose of minimizing pollution and utilizing manures from wastes for enhancing the soil productivity.

Organic farming envisages the comprehensive management approach to improve the soil health, eco-system of the region and the quality of product by using liquid organics such as Beejamruth, Jeevamruth and Panchagavya. Of these liquid organic manures, “Panchagavya” is more widely used as a traditional practice to safeguard plants, soil microorganisms and to increase plant production. This has shown beneficial effects on a variety of crops (Natarajan, 2002). In Sanskrit, Panchagavya means the blend of five products obtained from cow. The three direct constituents are dung, urine and milk and the two derived products are curd and ghee. These are a rich source of nutrients and act as a good source for the growth of microorganisms. When the above five products of the cow are suitably mixed and used, these have miraculous positive influence on living organisms (Swaminathan *et al*., 2007). Hence, application of Panchagavya in organic agriculture is not only increasing but also draws the attention of scientists to understand the process of making it. This helps to obtain consistent quality liquid organic manure. Making of Panchagavya is a microbial process that possibly involves microbial succession. Understanding the kind and number of microorganisms involved in its preparation is an important work for possibly wider application of panchagavya in agriculture.

Only 0.1-1% of all microorganisms observed in nature are known to be cultured under conventional laboratory conditions. While 99% remain abscure and the researchers are unable to study them even though they may have unique and potentially very useful abilities, such as; waste degradation or synthesis of compounds

like drugs or antibiotics etc. One of the recent approaches to study microbial diversity from environmental samples is 'Metagenomics'. Metagenome is a mixture of genomes of multiple organisms, particularly of mixed microbial genomes extracted from environmental samples like soil. Study of such metagenome is termed as metagenomics which is a new and powerful tool for accessing the untapped resources of biodiversity from ecosystem.

Metagenomics constitutes the functional and sequence based analysis of the collective microbial genomes (micro biome) from a particular environment or environmental niche. The technique is useful to study various microorganisms involved in a microbial process even without culturing them. Knowing the microorganisms involved in a process is a prerequisite for improving the process and also to develop methods to isolate and culture on a artificial media beneficial microorganisms from such samples.

The above-ground parts of plants are normally colonized by a variety of microorganism including bacteria, yeasts and fungi. The aerial habitat colonized by these microbes is termed as the phylloplane and the inhabitants are called epiphytes. For terrestrial plants, the phylloplane represents the interface between the above-ground parts of plants and the air. Most work on phylloplane microbiology has focused on leaves, a more dominant aerial plant structure. Conservative estimates indicate that roughly one billion square kilometres of worldwide leaf surfaces host more than 10^{26} bacteria, which are the most abundant colonizers of this habitat (Delmotte *et al*., 2009). A range of environmental factors such as temperature, rainfall, wind, and solar radiations are known to play an important role in determining patterns of bacterial phylloplane colonization (Kinkel, 1997). Much less is known of the role of plant factors in determining the diversity and dynamics of phylloplane microbial communities. Plant species (Kinkel *et al.,* 2000), gross plant morphology, the position and height of leaves (Thompson *et al*., 1993), and leaf age (Ercolani*,* 1991) have all been associated with enormous number of phylloplane bacterial populations.

Furthermore, various leaf surface features such as epidermal cell wall junctions (Davis and Barlansky, 1991) and grooves along the veins, stomata, and the base of trichomes (Mariano and Carter, 1993) and near hydathodes (Mew *et al*., 1985) have all been identified as preferential bacterial attachment sites, resulting in uneven

bacterial distribution in phylloplane. The composition of the phylloplane micro-biota has been analyzed in only a few studies by cultivation-independent methods (Lambais *et al*., 2006 and Yang *et al*., 2001); however, such methods are essential in the light of lack of knowledge on the yet uncultivated majority of bacteria existing in nature (Rappe and Giovannoni, 2003), or more specifically on plant leaves (Leveau, 2006). Not only their identity, but knowledge on other aspects in particular the physiological properties of phylloplane bacteria, their adaptations to the habitat and their potential role can be inhanced. So, the majority of knowledge of phylloplane microbiology has been gathered using culture in-dependent methods

With this background, a research proposal entitled “Metagenomic analysis of Panchagavya” was formulated with the following objectives in the present study:

1. Microbial analysis of Panchagavya through metagenomics approach
2. To assess the microbial changes in phylloplane of soyabean seedlings in response to application of panchagavya through metagenomic approach.

2. REVIEW OF LITERATURE

2.1 History and Meaning of panchagavya

Indian economy has been agriculture based since the vedic civilization. Cow and land were the major sources of income in the society. Almost all the oldest documents and mythological inscriptions were cow–centric. 'Ayurved' is one of the most ancient sciences of the world and in real sense it is derived from key words; '*Ayu*-means the life and veda-means the science. '*Ayurved'* thus is the science of life and not just a medical science. Ayurved has elaborately explained the secrets of healthy life style in the contexts of *Dinacharya* means "daily-routine"*, Rutucharya*-a description of seasonal regimen in Ayurveda. The qualities and use of milk, curd, ghee and other cow products are also included under daily needs. Also in the field of medicine, cow urine, ghee and cow dung have been extensively mentioned for the treatment of various health problems.

India is the second most populous country in the world. With the increasing population, the cultivable land resource is shrinking day by day. To meet the food, fuel, fodder and other needs of the growing population particularly after green revolution, intensive agriculture involving the use of chemical fertilizers in large amount has been practiced. On one hand this has resulted in manifold increase in the productivity of farm commodities on the other the adverse effects of these chemicals are clearly visible on soil structure, microflora, quality of water, food and fodder. Therefore, organic farming can be practiced as an alternative to maintain soil health, productivity besides keeping the environment safe (Rawat, 2002).

Organic farming envisages a comprehensive management approach to improve the soil health, eco-system of the region and the quality of produce. Liquid organics such as Beejamruth, Jeevamruth and Panchagavya are being indigenously prepared and used by farmers both as source of nutrients and as growth promoting preparations. Of these liquid organic manures, "Panchagavya" is most widely used to safeguard plants, soil microorganisms and to increase plant production. This has shown beneficial effects on a variety of crops (Natarajan, 2002). In Sanskrit, panchagavya means the blend of five products obtained from cow, namely; milk, curd, dung, ghee and urine. All these five products are individually called 'Gavya' and

collectively termed as panchagavya. Making of panchagavya is a microbial process that possibly involves microbial succession. Hence, understanding the kind and number of microorganisms involved in its preparation is an important aspect for wider application of panchagavya in agriculture.

The properties of panchagavya could be therefore attributed to the properties of the five ingredients used and the microbes involved in its preparation. In this chapter efforts have been made to compile the scientific literature on the use and application of panchagavya and the five panchagavya ingredients in agriculture.

2.2 Five ingredients of panchagavya

2.2.1 Cow dung

Nene (1999) reported that the cow dung had been used by Kautilya (321-296 BC), Varahamihira (505-587 AD), Surapala (1000 AD) and Someshwara Deva (1126 AD) in ancient history. It contained undigested fibre, epithelial cells, pigments and salts. It is rich in nitrogen, phosphorus, potassium, sulphur, micronutrients and intestinal bacteria.

Minhas *et al*. (1996) recorded that cow dung is composed of 82 per cent of water and 18 per cent of solid matter (minerals 0.1 per cent, ash 2.4 per cent, organic manure 14.6 per cent, Ca and Mg each 0.4 per cent, SO_3 0.05 per cent, Silica 1.5 per cent, N 0.5 per cent, P 0.2 per cent and K 0.5 per cent).

Plotnikova (1977) observed the presence of mycolytic bacteria in cow manure. The extract of cow dung compost showed the presence of bacterial isolates which inhibited the mycelial growth of *Fusarium oxysporum, Fusarium cucumerinum* and *Helminthosporium sigmoideum* (Kai *et al.,* 1990), suggesting that cow manure may be useful in obtaining products with antagonistic properties against plant pathogenic microorganisms. Cow dung is also an important ingredient used in compost making.

Aspergillus niger, Trichoderma harzianum, Bacillus cereus and *Bacillus subtilis* have been reported to be the major microbes in cow dung compost. When these microbes were co-cultured with the seedling blight inducing pathogens such as *Sclerotium rolfsii, Fusarium oxysporum, Pythium aphanidermatum, Helminthosporium*

maydis and *Rhizoctonia solani,* the mycelial growth of all tested pathogenic fungi was inhibited to the tune of 40-57 and 35.5-53.3 per cent, respectively by *Bacillus subtilis* and *B. cereus* (Muhammad and Amusa, 2003).

Thakur (2010) isolated fifty three bacteria from vermiwash and vermicompost, screened *in vitro* for various plant growth promoting traits. Only four out of 53 tested bacterial isolates showed antifungal activity against plant pathogens, *S. rolfsii, F. oxysporum* and *F. solani*. Therefore, cow dung has been traditionally used in the preparation of a number of useful products such as manures, compost and vermicompost which are applied to soil as source of nutrients and soil conditioners.

2.2.2 Cow urine

Reddy (1998) reported that cow urine (Gomutra) was rich in urea and acted both as a source of nutrient as well as a hormone. Cow's urine had 87 per cent water and 13 per cent solid matters consisting of minerals 1.4 per cent, ash 2.0 per cent, organic manure 6.0 per cent, Ca and Mg 0.15 per cent, SO_3 0.15 per cent, Silica 0.01 per cent, N 1.0 per cent, P traces and K 1.35 per cent. Urine also contained uric acid and hippuric acid in large quantities along with other minerals like NaCl, sulphates of Ca and Mg, potassium hippurate *etc*., (Singh and Kera 1994).

Menneer *et al*. (2003) have shown that cow urine application increased dry matter production of annual ryegrass by 85 per cent in treatments showing both light and moderately severe defoliations. Similarly, Gangaiah *et al*. (2004) have reported that the application of undiluted cow urine was detrimental to germination of maize crop, while application of 10 per cent of cow urine diluted with water enhanced the germination of maize progressively from 75 per cent to 100 per cent.

Joseph and Sankarganesh (2011) studied the antifungal activity of panchagavya and cow urine against soil borne pathogens. Sreenivasa and Naik (2011), using *in vitro* studies observed 92.1 and 57.3 per cent inhibition in mycelial growth of *Fusarium* spp. at 15 and 20 per cent concentrations of cow urine respectively.

Sathasivam *et al*. (2010) analyzed the antibacterial and antifungal activity of cow urine distillate against *Pseudomonas aeruginosa, Klebsiella pneumoniae,*

Aspergillus flavus and *A. niger.* The maximum growth suppression (7.06 mm in diameter) was observed in *A. flavus*.

Damodhar and Shinde (2010) carried out a study to know the effect of cow urine sprays on yield and quality of mango (*Mangifera indica* L.) cv. Alphonso. The results indicated that concentrations of cow urine used were 25, 35 and 55 per cent with three and six sprays. Six foliar sprays of 55 per cent cattle urine solution resulted in the highest fruit weight, volume, number of fruits, fruit yield per plant and total yield (tons/ ha). It is evident that cow urine has been used as a source of nutrient as well as a method to increase germination and yield of different crops.

2.2.3 Cow milk

Cow milk contains protein, fat, carbohydrate, amino acid, calcium, hydrogen peroxide, lactic acid and also Lactic Acid Bacteria (LAB) belonging to *Lactobacillus* (Graw, 1999). Shenoy *et al*. (2000) described the use of milk and observed that it changed the flower colour and enhanced the fruit taste of

Cow milk also had been used by farmers in ancient times and reported to be an excellent sticker and spreader (Casein) a good medium for saprophytic bacteria and as a virus inhibitor (Nene *et al*., 1999). According to Deshpande and Menon (1995) the general practice of sowing seeds involved soaking them in milk for ten days, taking out daily with hand, smearing with ghee, rolling many times in cow dung before the seeds were sown in soil. Such seeds grew and bloomed better when sprinkled with milk and water.

Masoud *et al*. (2011) analyzed milk and observed that *Lactococcus, Lactobacillus* and *Streptococcus*, have been the main bacteria. Pyrosequencing and DGGE analysis with 16S rDNA and cDNA obtained from cheeses were carried out. Their study indicated that these bacteria contributed towards viability and ripening of cheese. Other bacteria like *Corynebacterium*, *Halomonas, Pediococcus*, *Micrococcus* and *Staphylococcus*, which were encountered in some cheese samples at low percentages, have only been detected by pyrosequencing. This study indicated that 16S rRNA gene pyrosequencing is an efficient method for deep sequencing of microbial communities and it can expand our knowledge of the bacterial diversity in a product such as cheese obtained from raw milk.

Giannino *et al.* (2009) conducted culture independent analysis of milk and fresh curd samples of farms located in different valleys and altitudes. Their study indicated that for some Lactic Acid Bacteria (LAB) (*Streptococcus thermophilus, Enterococcus faecium, Enterococcus faecalis, Lactococcus lactis, Leuconostoc lactis*) alpine milk was a preferential niche for colonization. The microbiota included not only mesophilic and thermoresistant LAB but also adventitious bacteria (*Macrococcus caseolyticus, Rothia* spp.) and psychrotrophic bacteria (*Chryseobacterium* spp., *Pseudomonas* spp.), that were found initially in almost all samples, but disappeared after the warming up at 47–48°C of coagulated milk. Also, *Pantoea* spp. was primarily found in curds and only with a low incidence in milk samples, indicating its environmental origin. Finally, the sequencing data confirmed the presence of *E. faecium, E. faecalis and S. thermophilus* as major species present in curd. These species were also found in raw milk, clearly suggesting that milk is the original source of these diverse groups of fermentative bacteria.

2.2.4 Curd

LAB comprises of a heterogeneous group of genera that produce the end product lactic acid as a result of anaerobic respiration. They convert the milk into curd, predigest the milk proteins to potential hypotensive peptides, inhibit the growth of harmful and putrefactive microflora in the intestinal tract and promote the growth of beneficial bacteria needed for digestion (Beresford, 2001). Besides, LAB certain additional bacteria referred to as secondary microflora also influence the quality by altering the flavor, texture, consistency and organoleptic properties of curd. This secondary microflora can be non-starter lactic acid bacteria that develop during the process of fermentation showing a microbial succession in curd preparation.

Prasad (2012) found that the amount and strain of useful bacteria in curd vary from place to place and the number of species of *Lactobacillus* isolated from curd preparations in India is as high as 250 species per ml of curd. *Lactobacillus* is a heterogeneous group encompassing species with large variety of phenotypic, biochemical and physiological properties. Based on the mode of fermentation and type of end products, *Lactobacillus* has been classified into three major categories obligate homofermentative, facultative homofermentative and obligates heterofermentative

bacteria and reported curd as a source of very useful fermentative bacteria (Kenneth, 2008).

Lactobacillus and *Streptococcus* are considered to be the predominant species seen in curd (Gandhi and Natrajan, 2010). Their mutual stimulatory or associative action results in faster growth by utilizing limiting nutrients present in milk. They are able to grow in the presence of high oxygen content. Their interaction results in the production of small quantities of hydrogen peroxide which suppresses the growth of undesirable organisms. Unlike yogurt which specifically contains the interaction of *Lactobacillus delbrueckii* sub spp *bulgaricus* and *Streptococcus thermophilus* only, curd contains a mixture of various strains of LAB or yeast or combination of both the groups (Ghosh, 2012).

Conventionally, the main focus of lactic acid fermentation lies on the conversion of lactose into lactic acid by LAB. Besides, several other physico-chemical changes such as reduction in pH, production of diacetyl acetaldehyde and 2, 3, butylene glycol, conversion of milk fat into pre-digestible components, increase in levels of folic acid, niacin, pantothenic acid and vitamin B_6 and B_{12} also take place (Gandhi and Natrajan, 2010).

2.2.5 Microbes associated with fermented milk

Probiotics microorganism is a viable microbial dietary supplement that beneficially affects the host through its effects in the gastrointestinal tract. The characteristic features of LAB laid the foundation to one of the most extensively studied field of ‘probiotics’. Till date, all the probiotic bacteria known belong to LAB but all the LAB need not be probiotic in nature (Shruthy *et al*., 2011). The benefits imparted by probiotic strains on the host health and physiology are strain specific and therefore vary from strain to strain (Ghosh, 2012).

Nutrigenomics can act as a translational science that links food fermentomics, bacterial genomics and human metabolism (Vergères, 2013). Its application in studying the complex ecosystem, as in curd, can help in unraveling the diversity and dynamics of a multitude of microbial strains, their interactions with each other and essentially the entire food metabolome. It would be helpful to correlate the occurrence of certain microbial species and strains that impart desired flavour and sensorial traits to the fermented product. Microbes associated with milk fermentation are a distinct group with great potentialities to produce flavours, acids and several components which are known to influence metabolism.

2.3 Microbes associated with panchagavya

Solaiappan (2002) found that in panchagavya, population in term of colony forming units (CFUs) proven bio-fertilizer strains such as *Azospirillum* (10^{10}), *Azotobacter* (10^{9}), *Phosphobacteria* (10^{7}) and *Pseudomonas* (10^{6}) were found besides *Lactobacillus* per liter of panchagavya sample. Ammonia and nitrite oxidizers were found to colonize the leaves and increased the ammonia uptake and total N supply of spruce trees.

Swaminathan *et al.* (2007) reported the presence of naturally occurring beneficial microorganism's predominantly lactic acid bacteria, yeast, actinomycetes, photosynthetic bacteria and certain fungi in panchagavya. After fifteen days of incubation, the fermented product "Panchagavya" was used as a source for isolation of bacteria. The serial dilution and standard plate count method was used for isolation of Phosphorus solubilizing microorganisms which include largely bacteria and fungi. The most efficient phosphate solubilizing microorganisms isolated belonged to the genera of *Bacillus, Pseudomonas* and *Aspergillus*. The microorganisms in panchagavya are also known to secrete plant growth hormones that increased growth rates and improved yields of host plants.

2.4 Physico-chemical and biochemical properties of panchagavya

Somasundaram and Singram (2006) analyzed panchagavya to know its composition which was found to contain; total N (302 mg/kg), total P (218 mg/kg), total K (355 mg/kg), total sugars (205 mg/ml), glucose (6 mg/l), sodium (96 mg/kg), calcium (27 mg/kg), total organic carbon (0.8 per cent), IAA (9.15 mg/kg), GA (4 mg/kg),

phenols (0.75 µg/ml) bacteria (34 x 10^6cfu/ml), fungi (22 x 10^4cfu/ml), Actinomycetes (3 x 10^{12}cfu/ml), *Pseudomonas* spp (45 x 10^3cfu/ml), yeast (35 x 10^4cfu/ml), lactic acid bacteria (22 x 10^6cfu/ml), methylotrophs (5 x 10^3cfu/ml), *Azospirillum* (2 x 10^2cfu/ml), *Acetobacter* (43 x 10^3cfu/ml), ammonium oxidizers (24 x 10^5cfu/ml), nitrite oxidizers (2 x 10^2cfu/ml), pH (5.62), Zn (0.26 mg/kg), Fe (0.83 mg/kg), Mn (0.23 mg/kg) and Cu (0.2 mg/kg).

Similarly, Maheshwari *et al.* (2007) analyzed panchagavya in order to assess its physico-chemical and biological properties. They reported pH (5.45-6.46), total N (385-410 ppm), total P (230-255 ppm,), total K (345-370 ppm), organic carbon - (0.80-1.20%), Na (75.0-82.0 ppm), Ca (32-37 ppm), Mn (15-24 ppm), Fe (12-18 ppm), Zn (0.26 ppm), Mn (0.23 ppm), Cu (0.20 ppm), total sugars (165-255 ppm), reducing sugars (85-115 ppm), IAA (7.55-9.15 ppm), GA (3.50-4.50 ppm), phenols (0.75 mg/ml). The biochemical analysis of Panchagavya using GC-MS resulted in compounds of fatty acids, alkenes, and alcohols. Similarly, by culturing techniques population of *Lactobacillus* (22×10^6cfu/ml), Methylotrophs (5×10^3cfu/ml), *Pseudomonas* (45 × 10^3cfu /ml), total anaerobes (9–11.5×10^4 cfu/ml), bacteria (35–42×10^9 cfu/ml), fungi (13–16.5×10^4 cfu/ml), actinomycetes (6–9×10^2 cfu/ml), yeast (2–22.5×10^5 cfu/ml) were found in panchagavya.

Natarajan (2002) reported that increased yield of crop plants with panchagavya application was due to enhancement in the biological efficiency of crop plants. Presence of beneficial bioactive compounds such as GA_3, IAA etc., in panchagavya and increased level of phosphorus and potassium and other essential nutrients present in compost were known to increase yield (Somasundaram, 2003).

Suresh *et al*. (2011) studied panchagavya's biochemical properties. The results showed that panchagavya had slightly acidic pH, increased EC, rich in macro, micro nutrients and organic carbon. Many effective microorganisms were isolated from panchagavya viz., bacteria, fungi, actinomycetes. Particularly *Pseudomonas*, Lactic acid bacteria, *Azospirillum* and *Azotobacter* were predominant. Panchagavya did not have direct antimicrobial activity against microorganisms.

2.5 Applications of Panchagavya and response of plants

2.5.1 Effect of panchagavya on growth, yield and yield parameters of various crops

Studies conducted at Indian Grassland and Fodder Research Institute (IGFRI), Jhansi with various Jaivik and Vedic Krishi inputs such as Angara, Amrutpani, Panchagavya and Gomutra, *etc*., indicated that all these products improved crop productivity, microbial population and activity in soils. Growth of mustard was found to increase due to the foliar application of Gomutra and panchagavya. Lucerne fodder yield was also found to significantly increase due to application of Gomutra and panchagavya as seed soaking treatment. Panchagavya when applied twice increased the productivity by 49.63 per cent over control. Application of Angara to soil and panchagavya as foliar spray was also found to increase sorghum grain and dry fodder yields (Veerabhadraih *et al*., 2006).

Shwetha and Babalad (2008) conducted an experiment to know the effect of nutrient management through organics in soybean and wheat cropping system at Main Agricultural Research Station, Dharwad on a medium deep black clay loam soil. They reported that significantly higher leaf area index (LAI), plant height, number of branches, dry matter accumulation, seed yield and other yield parameters like number of pods per plant were recorded in treatments with the application of organic manures in combination with fermented organics viz, Beejamruth, Jeevamruth, Panchagavya compared to application of organics alone.

Vennila and Jayanthi (2008) reported that application of 100 per cent recommended dose of fertilizers along with panchagavya spray @ 2 percent significantly increased the number of fruits per plant, fruit weight (g) and fruit yield (q ha^{-1}) of okra.

In the study by Sadar *et al*. (2011) panchagavya treatment was found to be significant in improving the seed germination in soybean (*Glycine max*) over uninoculated control. The maximum germination of 83 per cent was recorded in treatment with 4 percent panchagavya followed by other in the order 50 per cent germination in treatment with 2 per cent panchagavya, 46 per cent with 6 per cent Panchagavya treatment and 33 per cent in 8 per cent panchagavya treatment.

However, all the treatments showed an improvement in germination, over uninocalated control in which the germination was only 10 per cent. In treatment with 10 per cent panchagavya the germination was absent.

Rajesh and Kaliyamoorthy (2013) conducted a field experiments to find the variation in growth, biochemical and yield parameters of black gram under different concentrations (0, 1, 3, 5, 7.5 and 10 per cent) of panchagavya, and all the parameters were found to increase in treatment with 3 per cent panchagavya application. Since, there was increase in growth and yield at low concentration of panchagavya. In this study it was recommended that the panchagavya be used for spray after diluting.

Krishnapriya *et al*. (2011) designed an experiment to study the yield and biochemical contents of rice in response to panchagavya (an organic fertilizer) application. Panchagavya was prepared and applied through flow irrigation on the 15^{th}, 30^{th}, 45^{th} and 60^{th} day after transplanting. Shoot length, height of the plant, number of grains, 1000 grains weight, number of spikelets, grain protein and carbohydrate content were studied. The results showed that panchagavya had a positive influence over the control in all parameter studied.

2.5.2 Effect of panchagavya on quality parameters of crops

Hannah *et al.* (2005) observed that the panchagavya spray produced tastier banana fruits at the Agricultural College Research Institute, Tamil Nadu. In this study panchagavya was sprayed to banana @ 3 per cent which resulted in improvement in quality of fruits *viz.,* total soluble sugars, total sugars and reduced the negative quality characters like acidity and ascorbic acid.

Dipping the chilli seedling roots in Beejamruth along with soil application of Jeevamruth (500 l ha^{-1}) at 10 DAT and foliar application of panchagavya @ 3% at the time of flowering recorded higher ascorbic acid and capsaicin content in chilli fruits (Sreenivasa *et al.,* 2010). In another study organically grown tomato contained higher vitamin C, vitamin A and potassium over conventionally grown tomato (Pither and Hall, 1990).

Sanwal *et al.* (2005) noted that foliar spray of panchagavya and amrutpani at 10 per cent and use of natural growth promoters resulted in higher dry matter content, pH, ascorbic acid content, total soluble solids, reducing sugars and total sugars, but lower fibre contents in stems and flowers of broccoli than those grown with NPK treatment.

Kumawat *et al.* (2009) reported that application of panchgavya + neem leaf extract at branching and flowering stage of crop was advantageous in increasing chlorophyll content, physiological growth, nutrient content and uptake, dry matter accumulation, yield and yield attributes and economics of groundnut.

Panchagavya was evaluated for its antioxidant potential by HPTLC-DPPH bio-autography method as well as assays for Ferric reducing antioxidant power (FRAP), DPPH-free radical scavenging activity and Superoxide radical scavenging activity. HPTLC-DPPH bio-autography study revealed the presence of several antioxidant compounds in panchagavya. In all the assays performed, considerable antioxidant activity was observed. On comparison of the data of three different batches of the samples studied, it showed 98.3 - 99.8% correlation with total phenolic content, FRAP and DPPH assay (Athavale *et al*., 2012).

2.5.3 Influence of panchagavya on seed germination and seedling vigour

Palekar *et al*. (2006) reported that beejamruth is not only a source of nutrients, but it is a product which contains ingradients *viz*., cow dung (5 kg), cow urine (5 litres), lime (50 g) and 20 litres water. It is being used by the organic farmers for seed or seedling treatment which was found to increase seed germination and seedling growth as it contained growth hormones and beneficial microflora.

Sreenivasa and Naik (2011) reported that the beneficial microorganisms present in Beejamruth produced IAA and GA and resulted in improvement in seed germination, seedling length and seed vigour in soybean.

Nagaraj and Sreenivasa (2009) conducted an experiment at the Institute of Organic Farming, UAS, Dharwad to study the influence of bacterial isolated from Panchagavya on seed germination and seedling vigour in wheat. On 8^{th} day after sowing, significantly the highest percentage of seed germination (99 percent) was

noticed in the seeds treated with bacterial culture PB9 and PB15 while the lowest germination was recorded in uninoculated seeds 85 percent which indicated positive role of bacterial isolates of panchagavya in promoting seed germination. The bacterial culture PB9 registered significantly higher seedling length (28.5 cm) and vigor index (2822) while these parameters were markedly the lowest in the control (16.5 cm and 1403, respectively).

2.5.4 Effect of panchagavya on nutrient content of crops

Poul *et al.* (2004) conducted an experiment to study the effect of organic and inorganic nutrient sources on the growth, yield and nutrient uptake by tomato in a calcareous vertisol at Marathwada Agricultural University, Parbhani during 1997-98. The results revealed that the application of organics such as cow dung, urine slurry and zatpatkhat along with inorganics recorded higher uptake of N, P and K (*i.e.*, 1.25 to 1.26 g N per plant, 0.07 to 0.08 g P_2O_5 per plant and 0.84 to 0.86 g K_2O per plant) than with the application of recommended dose of fertilizers (1.05, 0.06 and 0.8 g/plant N, P_2O_5 and K_2O respectively).

Shwetha and Bablad (2008) reported that the soil organic carbon content and available soil nutrients *viz.*, N, P_2O_5 and K_2O after harvest of soybean and wheat were significantly higher with the application of organic manures alone or in combination with fermented liquid organics as compared to the application of organics alone. Similarly, the uptake of N, P and K were more with the combined application of fermented liquid organics and organics as compared to the RDF + FYM and the individual application of organics and fermented liquid organics. The organic carbon, available N, P_2O_5 and K_2O values after harvest respectively ranged respectively between 0.72 - 0.74 percent, 263.4 - 269.6 kg N per ha, 17.5 to 17.9 kg P_2O_5 per ha and 383-391 kg K_2O per ha.

2.6 Role of panchagavya in plant disease management

Sugha (2005) evaluated antifungal potential of panchgavya against *R. solani, S. rolfsii, F. solani,* and *Phytophthora colocasiae* and advocated that the dipping of mycelial bits for 6 h in panchgavya caused complete suppression of mycelial growth of *R. solani* and in other pathogens, the growth inhibition ranged between 88.1 - 92.3 per cent.

Basak and Lee (2005) conducted an experiment to study the efficacy and *in vitro* activities of cow urine and dung for controlling wilt caused by *F. oxysporum* f.sp. *cucumerinum* of cucumber and *F. solani* f.sp. *cucurbitae.* Cow dung solution showed 80-84 per cent inhibition of wilt pathogens and cow urine showed 100 per cent inhibition of wilt pathogens.

Joseph and Sankarganesh (2011) studied the antifungal activity of panchgavya and cow urine against soil borne pathogens. Sreenivasa and Naik (2011), observed 92.1 and 57.3 per cent inhibition in mycelial growth of *Fusarium* spp, at 15 and 20 per cent concentrations of cow urine *in vitro.*

Sireesha *et al*. (2013) conducted field trials for control of *Pyricularia grisea* in Rice, c.v. NLR -34242. The treatments were; Neem seed kernel extract, Neem cake, Neem oil, Panchagavya, *Pseudomonas fluorescens, Trichoderma viride* and *Pongamia pinnata.* All the treatments showed significant control of pathogen. *Pseudomonas flourescens* was found to be the most effective in controlling the leaf blast and neck blast incidence and also increased grain yield and number of filled grains per plant.

2.7 Extraction and purification of secondary metabolites from panchagavya

Extraction of the metabolites is generally carried out either by solvent-solvent extraction or extraction using salt precipitation. Organic microbial metabolites are soluble in organic solvent. But their solubility differs from solvent to solvent. Selection of a particular solvent which can dissolve almost all the organic compounds present in the secondary metabolites is necessary (Vijayakumar *et al*., 2012). Generally used solvents include ethyl acetate, butanol, chloroform, methanol etc. of which, ethyl acetate seems to be the most common. For extraction using salt precipitation, (Khan and Patel, 2011), used ammonium sulphate precipitation and observed that up to 70 percent saturation was required in order to precipitate the proteineceous metabolites.

Purification of the secondary metabolites is a step by step process involving several chromatographic techniques. Thin Layer Chromatography (TLC), is a simple and most commonly used technique for separation/fractionation of the metabolites from a microbial strain. Staining with different chemical reagents like anisaldehyde/H_2SO_4, orcin-$FeCl_3$, vanillin/H_2SO_4, iodine vapour, ninhydrin, Eldrich reagent, naphthoresorcin-sulphuric acid etc., would help in visualization of the complete secondary metabolite pattern of a microbe (Solanki *et al*., 2013). Different organic solvents were like methanol, butanol, ethyl acetate, chloroform, n-hexane, acetic acid along with water were mixed in different ratios and used as mobile phase. Hemashenpagam, (2011) extracted secondary metabolites from 25 different isolates of actinomycetes. The crude extracts were further analysed by TLC on silica plates. Charecterization of the Rf values in the TLC chromatograph revealed that metabolites from two of the *Streptomyces* species; sp. 2 and 3, were under aminoglycoside group, very much related to Streptomycin and Cephalexin respectively. Column chromatography is another commonly used technique for fractionation of the secondary metabolites (Elnagger *et al*., 2001). Different fraction developed can be separated, eluted and used for further analysis. Use of column chromatography for purification of microbial metabolites was also reported by Sunaryanto *et al*. (2010).

The identification of active compounds, especially new compounds from fermentation samples (extracts, whole broths) is said to be one of the most complicated, labour intensive, time consuming steps of the screening protocols (Berdy, 2005). In general, the crude extracts are evaluated by chemical screening or by various biological and pharmaceutical screening approaches. The latter mostly focussed on the compounds with highly sensitive biological activity. These approaches, however, are likely to overlook novel compounds which may be active against other targets. Identification of a known or an unknown compound requires separation of the individual compound from the crude extract. It should be followed by screening of each separated compound for the bioactivities. Most often, a failure in separation and identification of the bioactive compounds completely is attributed to the lack of technical inputs. Recently, a great progress in the robotic and automated methods in chromatographic isolation methods [Liquid chromatography mass spectrometry (LC-MS), Liquid chromatography mass spectrometry-Evaporative Light Scattering Detector (LC-MS-ELSD), Gas Chromatography-Mass Spectrometry

(GCMS), High pressure liquid chromatography- equipped with ultra violet and visible light detector (HPLC-UV-VIS), High Pressure Liquid Chromatography- Evaporative Light Scattering Detector (HPLC-ELSD), High Pressure Liquid Chromatography-Photodiode Array Detectors (HPLC-PDA), High Performance Counter-Current Chromatography (HPCCC), Capillary Zone Electrophoresis (CZE), etc.] and spectroscopic identification techniques (multi-dimensional NMR, X-Ray crystallography, NOESY, BMS, electrospray MS, HR-MS) more or less fullfill the requirements of the modern HTS (high through-put screening) tasks (Berdy, 2005). These recent techniques singly or in combination are helpful to understand and detect an array of microbial metabolites.

2.8 Isolation, purification and PCR amplification of high molecular weight microbial community DNA extracted from panchagavya

2.8.1 High molecular weight microbial community DNA isolation

Metagenomic approaches require access to high quality DNA, in sufficient quantity to understand microbial ecology (Vogel *et al*., 2009). DNA extraction is the key step of metagenomic approaches (Frostegard *et al*., 1999; Lakay *et al*., 2007 and Delmont *et al*., 2011). Total genomic DNA (metagenome) derived from microbial communities can provide complementary information about the overall community structure (“species” composition) and the total genetic diversity (Ritz *et al,*. 1997; Johnsen *et al*., 2001). A critical methodological aspect of DNA extraction is the DNA yield especially when large quantities are needed for high throughput sequencing or cloning (Delmont *et al*., 2011). Extraction of pure nucleic acids from environmental samples has been a challenge because of the complex and heterogeneous nature of the sediment and the inhibition of biochemical reactions by co-extracted substances such as humic acids (Young *et al*., 1993). Humic substance refers to organic matter that has been decomposed by bacteria, fungi and protozoa to the final point where it is resistant to further breakdown. These substances share physico-chemical similarity with that of nucleic acids and hence are known to inhibit the activity of basic enzymes of molecular biology like that of polymerases and restriction endonucleases (Porteous and Armstrong, 1991; Tsai and Olson, 1992), thereby interferes with various

downstream processes like cloning, sequencing *etc*. Hence, major focus while isolating metagenomic DNA is to obtain high quality of purified DNA, which is free from humic substances. Various protocols have been developed for obtaining purified DNA but individually cannot be used as the standard protocol, as the output varies for different types of sediment samples. A combination of different available protocols can be used to obtain high molecular weight community DNA.

2.8.2 Direct method of DNA extraction

Metagenomic approaches for DNA isolation can be separated in to two general strategies. The first strategy, which is more commonly used, is direct DNA extraction which is done by direct cell lysis within a sample (Ogram *et al*., 1987 and Van Elsas *et al*., 1997). The second strategy is indirect DNA extraction where cells are first removed from a sample and then are subsequently lysed for DNA extraction (Jacobsen and Rasmussen, 1992; Berry *et al*., 2003). Indirect and direct DNA extraction methods access similar levels of microbial biodiversity; however they do not access exactly the same microbial populations. In spite of a lower purity, the DNA yield in terms of mass of DNA per mass of sample is greater with indirect than direct extraction (Steffan *et al.,* 1988 and Leff *et al*., 1995), although the direct method access greater genetic diversity in sample in spite of the low yield. The direct DNA extraction does not appear to be particularly more biased than the indirect approach, and thus, is a useful approach for covering in-depth microbial community (Delmont *et al*., 2011).

In the last couple of decades, various direct extraction methods have been developed like chemical and enzymatic lysis by Zhou *et al*. (1996), additional physical cell disruption methods such as freeze thaw cycles (Cho *et al*., 1996), freezing followed by grinding (Volossiouk *et al.,* 1995) or bead beating (Miller *et al.,* 1999) where physical disruption of cells is done by glass or ceramic beads, the efficiency of cell disruption and damage to DNA depends on the type and speed of beads. Even under optimized conditions, bead beating results in severe fragmentation of DNA, as compared to that in chemical or freeze-thaw lysis. A hot-lysis method was developed by Selenska and Klingmuller (1991). More popular is the detergent treatment which includes SDS at 1- 2% concentration and salt concentration of 1M or more and lysis is performed by heating coupled with shaking (Edgcomb *et al*., 1999). High community

and enough purity microbial DNA is very essential for the further downstream processing in metagenomic analysis.

2.8.3 Purification of high community DNA

Panchagavya also contains organic matter in addition to DNA, a major fraction of which interferes with various molecular methods (Robe *et al*., 2003; Whitehouse and Hottel 2007) hindering DNA extraction from panchagavya. The extracted microbial community DNA from panchagavya hence was unfit for further analysis, which is the actual objective of any metagenomic approach. Humic substances are a major component of organic matter. Though the chemical composition is highly complex, these compounds readily co-purify with DNA and are difficult to remove without additional, laborious and time intensive treatments to obtain DNA suitable for PCR (Romanowski *et al*., 1992). Several methods have been developed to remove these inhibitors from the organic samples and to obtain purified DNA, these include Sephadex spin column (Miller *et al*., 1999), addition of polyvinyl polypyrrolidone (PVPP) (Zhou *et al*., 1996), addition of hexadecyltrimethyl ammonium bromide (CTAB), addition of bovine serum albumin (BSA) (Malik *et al*., 1994; Fortin *et al*., 2004), size exclusion chromatography (Erb and Wagner-Dobler, 1993; Kuske *et al*., 1998; Leff *et al*., 1995; Hurt *et al*., 2001) and agarose gel electrophoresis followed by excision and DNA extraction from gel matrix (Malik *et al.,*1994; More *et al*., 1994).

Use of multivalent cations has been a standard method for removing suspended organic solids during the purification of drinking water; a process commonly referred to as chemical flocculation. In theory, these chemicals could remove organic inhibitors via flocculation during sample lysis and homogenization, potentially eliminating or reducing the need for additional treatments and the associated sample loss. Braid *et al.,* (2003) investigated chemical flocculation using multivalent cations as a potential method for eliminating inhibitors during the extraction process. The solutions of aluminium ammonium sulphate ($AlNH_4(SO_4)_2$), calcium chloride ($CaCl_2$), ferric chloride ($FeCl_3$) and magnesium chloride ($MgCl_2$) during extraction showed significant reduction in the co-purification of inhibitors with minimal loss of DNA yield.

2.8.4 PCR amplification of DNA extracted from panchagavya

The information in nucleic acids of microorganisms can be used to investigate and compare diversity at different organization levels, ranging from variability within species to diversity of communities (Johnsen *et al*., 2001). Among nucleic acid-based approaches in metagenomics, most methods in use currently in one way or another on the polymerase chain reaction (PCR) (Albuquerque *et al*., 2009). PCR conditions need to be optimized before going for analysis of new microbial community and whenever new primers are used. Most commonly optimized variables are primer annealing temperature, concentration of polymerase enzyme, cofactor Mg^{2+} ions and template concentration (Boleda *et al.,* 1996). Nucleic acid quantification is one of the most important steps before performing PCR so as to ensure PCR amplification. Dilution of template can be effective in reducing concentrations of contaminating substances which inhibits PCR mostly in case of organic sample DNA. Modified methods like touchdown PCR (Muyzer *et al*., 1993) and nested PCR (Nakatsu *et al*., 2000) can be used to increase specificity and efficiency of PCR.

For analysis of microbial diversity, selection of marker genes should be done on the basis of information supplied and composition of gene. Diversity analysis done for sequences which comprise of both conserved region and variable regions are desired. Majority of community studies target gene which provides taxonomic and phylogenetic information like that of conserved 16S ribosomal RNA gene, which can be used for PCR amplification from environmental DNA with universal oligonucleotide primers (Albuquerque *et al*., 2009). Various rRNA specific primers are also available for discrimination of communities at different phylogenetic levels such as for eubacteria (Watanabe *et al.,* 2001), eukaryotes (Hannen *et al.,* 1998) and archaea (Nakatsu *et al*., 2000). In general, the partial sequences-based phylogenetic analysis is largely consistent with 16S rRNA gene (Rath *et al*., 1998). The partial 16S rRNA gene fragment is able to distinguish most phylotypes from their relatives and determine phylogenetic relationships (Cebron *et al,*. 2004; Muyzer *et al*., 1993).

Hence, a combination of specific or universal primers for eubacteria and standardizing PCR conditions from important parts of most metagenomic analysis.

2.9 Denaturing Gradient Gel Electrophoresis (DGGE)

For examining sample microbial population and functions, different molecular methods have been developed, The PCR-DGGE method targeting the 16S rRNA gene is most widely applied for the studies on bacterial community structure in the environment. This is because; this gene is essentially helpful in tracing phylogenetic relationships of prokaryotes (Muyzer, 1999).

The DGGE has been widely used to study the dynamics and structure of complex microbial communities. This molecular approach has been very useful and has provided the significant progress in understanding microbial diversity of natural ecosystems like soil (Prosser, 2002). However, both the over and underestimation of real diversity may occur due to inherent methodological limitations of this PCR-dependent method (Fromin *et al*., 2002). This limitation can be overcome by coupling the DGGE with the sequencing, which can help in avoiding the incorrect interpretation of the profiles due to the presence of different phylogenetic affiliations at the same gel position (More, 2011).

DNA fingerprinting has been widely used for studying soil bacterial diversity. Several fingerprinting techniques have been developed to provide a rapid assessment of microbial communities, particularly for comparison and monitoring purposes. Soil microbial communities have also been characterized using DGGE (Handelsman, 2004).

The diversity of soil microbial communities and their composition can be provided by DNA based techniques, since they survey both the cultured and often predominant uncultured members of the community (More, 2011). Surveys of soil bacteria by PCR amplification of 16S rRNA genes followed by cloning and sequencing have shown that soil bacterial communities are extremely diverse and may contain abundant uncultured representatives of novel, undescribed bacterial divisions (Hugenholtz *et al*., 1998). However, due to complexity of microbial communities and some of the inherent methodological limitations, the uses of culture independent DNA-based approaches face some significant shortcomings at the present time. A major shortcoming is a strong dependence of the data obtained on the protocol used for extraction of nucleic acids from environmental samples (Delmont *et al*., 2011).

However, such limitations can be sidestepped today by the considerably increased amount of sequence data relevant to the structure, dynamics and functions of bacterial population in sample which are delivered using metagenomics and deep-sequencing approaches (Metzker, 2010). Strategies adopted for environmental sample metagenomic studies are shot-gun metagenome sequencing, amplification and sequencing of 16S rDNA. Early metagenomic projects used Sanger sequencing and were costly, but the advent of next-generation sequencing technology, such as Roche's GS-FLX Genome Sequencer (Roche Diagnostics Ltd., Burgess Hill, West Sussex, UK), has made large scale metagenomic studies more practical and cost-effective (Adams *et al*., 2009). So, high-throughput sequencing and microarrays in conjunction with conventional methodologies can reveal the relationship between microbial community function and phylogenetic diversity, providing greater insight into the consequences of environmental change on ecosystem function

2.9.1 Methods to analyze DGGE pattern

Community-level molecular techniques are widely used in comparative microbial ecology to assess the diversity of microbial communities and their response to changing environments. These include denaturing and temperature gradient gel electrophoresis (DGGE/TGGE), single strand conformation polymorphism (SSCP), length heterogeneity-PCR (LH-PCR), terminal-restriction fragment length polymorphism (TRFLP) and 16S rRNA gene clone libraries. Among these techniques, DGGE is one of the most well established molecular tools in microbial ecology (Boon *et al*., 2002). The amount of data derived from these techniques available in literature is continuously increasing and the lack of a universal way to interpret the raw fingerprint itself makes it difficult to compare between different results. Taking the DGGE technique as an example, it may be proposed that independent theoretical interpretation of the DGGE pattern, would be based on a straightforward processing on three levels of analysis: (i) the range-weighted richness (*Rr*) reflecting the carrying capacity of the system, (Apajalahti *et al*., 2001) (ii) the dynamics (*Dy*) reflecting the specific rate of species coming to significance, (Wittebolle *et al*., 2005) and (iii) functional organization (*Fo*), defined through a relation between the structure of a microbial community and its functionality (Mertens *et al*., 2005). Profiles of DGGE were analyzed in another three ways. (i) Shannon-Wiener index (also known as the Shannon-Weaver index) to check bacterial diversity (Boon *et al*., 2002). (ii) A

hierarchical cluster analysis to analyse the banding patterns and expressed as a dendrogram. (iii) Logistic regression analysis to compare individual DGGE bands and their intensities (Fromin *et al*., 2002; Smalla *et al*., 2001). Studies using molecular techniques such as DGGE have demonstrated that a unculture-based approach can severely underestimate the bacterial diversity in most environments.

2.10 High-Throughput sequencing technologies

Culture independent analyses arose to overcome the limitations of the classical approach and have been extensively used in food, agriculture and environmental microbiology (Quigley *et al.,* 2011 and Cocolin *et al.,* 2011). The scope of microbial analysis can depend on the sample of our interest and the target microbes could be pathogens, spoilage associated or beneficial microorganisms. Such microbial population involved can now be studied by using High-Throughput Sequencing (HTS) approaches after direct nucleic acid extraction from the sample of interest. Different hypervariable regions of 16S rRNA gene were amplified and used for sequencing to study microbiota of taxonomic interest. It is the most common method followed for HTS technology (Ercolini, 2013).

Girija *et al.* (2013) extracted total microbial community DNA from fresh cow dung and bacterial 16S rDNA genes were subsequently amplified, cloned and deposited in Gene Bank. From the results, it was found that bacteria belonging to phyla Bacteroidetes (38.3%), Firmicutes (29.8%), Proteobacteria (21.3%) would be detected using this approach.

Zhang *et al.* (2014) have selected natural mature forest and natural secondary forest and analyzed the soil microbial community and metabolic potential combining the high-throughput sequencing and GeoChip technologies. Phylogenetic analysis based on 16S rRNA sequencing showed that one unknown archaeal phylum and 15 known bacterial phyla as well as unclassified phylotypes were present in these forest soils and Acidobacteria*,* Protecobacteria and Actinobacteria were the three most abundant phyla. The detected microbial functional gene groups were related to different biogeochemical processes, including carbon degradation, carbon fixation, methane metabolism, nitrogen cycling, phosphorus utilization, sulfur cycling etc. Although the results can't directly reflect the actual microbial populations and

functional activities, this study provides insight into the potential activity of the microbial community and associated feedback responses of the terrestrial ecosystem to environmental changes.

Das *et al*. (2013) studied the bacterial community structure and abundance in agricultural soils with varying levels of arsenic contamination at Ambagarh Chauki block, Chhattisgarh, India, based on polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of the 16S rRNA gene and the most probable number-polymerase chain reaction (MPN-PCR). The results revealed that the bacterial communities of arsenic-contaminated soils were dominated by *β-proteobacteria* (36%), *γ - proteobacteria* (21%), *δ-proteobacteria* (11%), *α-proteobacteria* (11%) and Bacteroidetes (11%). The bacterial composition of high arsenic-contaminated soils differed significantly from that of low arsenic-contaminated soils. The *Proteobacteria* appeared to be more resistant to arsenic contamination, while the Bacteroidetes and *Nitrospirae* were more sensitive to it.

Thoetkiattikul *et al*. (2013) characterized the microbial profiles of rumen fluid obtained from dairy cows fed on three different fiber/starch diet compositions. Tagged 16S rRNA gene pyrosequencing and statistical analysis revealed that the dominant ruminal bacteria shared by all three sample groups belonged to phyla Bacteroidetes, Firmicutes, and Proteobacteria. However, the relative abundance of these bacterial groups was markedly affected by diet composition. In animals fed with a high fiber diet, the fibrolytic and cellulolytic bacteria *Lachnospiraceae, Ruminococcaceae,* and *Fibrobacteraceae* were found with the highest abundance compared to animals fed with other diets lower in fiber content. The polysaccharide-degrading bacteria belonging to *Prevotellaceae* and *Flavobacteriaceae* bacteria were the most abundant in the rumen of cows fed on diet with the highest starch content. These data highlight the ruminal microbiome's ability to adapt to feed composition.

Davinic *et al*. (2012) collected soil samples from the Texas High Plains region under a variety of dry land and irrigated cropping systems. The soil was separated into macroaggregates, microaggregates, and silt + clay fractions. Also the soil was analyzed for bacterial diversity via pyrosequencing of the 16S rRNA gene and SOC quantity and quality using a combustion method and mid-infrared diffuse reflectance spectroscopy (mid-IR), respectively. Results from pyrosequencing showed that each

soil microenvironment supported a distinct bacterial community. Similarly, mid-IR data revealed distinct spectral features indicating that these fractions were also distinguished by organic and mineral composition. This study integrated physical, chemical, and molecular techniques to assess relationships between soil bacterial community structures and the quantity and quality of soil organic carbon (SOC) at the soil microenvironment scale (e.g., within different aggregate size-fractions). This is the first study to investigate soil bacterial communities among soil aggregates using pyrosequencing and to associate these communities to specific soil carbon chemistries as indicated by mid-IR absorbance.

From the published literature it is evident that molecular techniques such as DGGE coupled with pyrosequencing are effectively employed to understand microbial diversity particularly of bacterial diversity from diverse ecosystems.

3. MATERIAL AND METHODS

A study was conducted to understand the microbial succession in panchagavya making, following metagenomics approach and to characterize it biochemically and microbiologically. The materials used and methods followed in this study are presented in this chapter.

3.1 Ingredients of panchagavya

Five ingredients essential for panchagavya preparation namely; cow dung, cow urine, cow milk, curd and ghee made from cow's milk were either freshly collected or prepared. Cow dung, urine and milk were freshly collected from the dairy unit of Institute of Organic Farming (IOF), University of Agricultural Sciences (UAS) Dharwad. Preparation of curd was done using the whole cow milk obtained from Dairy Farm, IOF, UAS, Dharwad. Initially, the milk was boiled and allowed to cool at room temperature. Prior to inoculation, 500 ml specimen bottles (Tarsons) were washed, dried, autoclaved and exposed to UV for 20 minutes. Into each such bottle 250 ml boiled, cooled milk was poured under aseptic conditions. To this milk the starter curd sample 25 ml was added stirred and kept at room temperature for 8 hours. Curd was prepared under suitable environmental condition and brought to laboratory. Fresh ghee for panchagavya preparation was procured from the commercial outlets of Karnataka Milk Federation (KMF).

3.1.1 Preparation of panchagavya under laboratory conditions

In the present study, Panchagavya was prepared using the protocol given by Swaminathan *et al*. (2007). The ingredient quantity was reduced to 1/10th volume of original protocol with minor modifications. Accordingly, 600 g of cow dung and 100 g of ghee were mixed thoroughly and kept for two days. Then, on 3rd day, 400 ml of cow urine and one litre of water were subsequently added, stirred and left for another 12 days. Finally, on 15th day, other constituents like sugarcane juice 300 ml, 200 ml of cow's milk, 200 ml of curd, 200 ml of coconut water, 25 g jaggery and one ripened banana were added and mixed thoroughly. The contents were kept at room temperature for another 15 days, with intermittent mixing clock wise and anti-clock

wise direction twice a day. The preparation was carried out in 5 litre glass bottles under ambient conditions.

3.1.2 Collection of panchagavya samples at 7 days interval and extraction of secondary metabolites

Panchagavya samples were collected at every 7 days interval to extract secondary metabolites. Extraction of various secondary metabolites was carried out using solvent- solvent extraction method given by Sharma and Parihar (2010). At each stage, 100 ml of panchagavya mixture was collected and centrifuged at 14,000 rpm for 15 minutes to remove the solids. The supernatant was aseptically filtered and collected into sterile bottle. The filtrate was mixed with ethyl acetate in 1:1 (v/v) ratio and shaken vigorously overnight in a rotary shaker. The solvent phase that contained various volatile compounds was separated from the aqueous phase using a separating funnel. Then, the solvent phase was evaporated to dryness using a rotary vacuum evaporator (Heidolph) and the residue were dissolved in methanol (HPLC grade) before subjecting it into GCMS analysis.

3.1.3 Gas Chromatography Mass Spectroscopy (GCMS) analysis

Chemical screening of the active compounds present in the crude extracts was done using GCMS (Thermo Scientific). The GCMS analysis was done by outsourcing to a facility available at the Indian Institute of Sciences (IISc.) campus, Bengaluru. The analysis was conducted according to the following temperature program: 40°C maintained for 2 min: increased @ 10°C min^{-1} up to 300°C, and eventually maintained for 5 min. The ions were detected in the range 30-600 m/z (mass to charge ratio). The software used for data analysis was AMDIS, while NIST 11 library was used for comparison.

3.2 Extraction of microbial community DNA from panchagavya

Before extracting DNA from Panchagavya, the sample was thoroughly shaken and passed through a four layer of muslin cloth, to remove undigested plant material present in dung. Direct method of DNA extraction (Kim *et al*., 2010) was used for isolating microbial community DNA from panchagavya, with slight modification which are described below. The modifications were done for pH and concentration of the

buffer components along with addition of 100 mM EDTA (Ethyl diamine tetra acetic acid). For removal of humic acid inhibitors, chemical flocculation was done using 100 mM volume added per unit sample concentration of $CaCl_2$ as chemical flocculant.

Metagenomic DNA was extracted as described previously, with some modifications to procedures outlined by Zhou *et al*. (1996); Kim *et al*. (2010); Kwon *et al.* (2010). Panchagavya sample was filtered through four layered muslin cloth. Five ml of filtered panchagavya sample was added to 13.5 ml of the DNA extraction buffer, composition [100 mM Tris HCl (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM sodium phosphate buffer (pH 8.0), 1.5 M NaCl, 1% hexadecylmethylammonium bromide (CTAB)] and incubated for 1 h at 37 °C with rotary agitation at 50 rpm. To the content 50 µl of Proteinase K, at a concentration (20 mg/ml) was added into the filtrate and incubated for 1 h at 37 ºC under shaking condition at 120 rpm. To this mixture 1.5 ml of 20% SDS solution was added and incubated at 65 ºC for 2 h in a water bath with intermittent mixing every 20 min. Further, the contents were centrifuged for 10 min, at 10000 rpm and the supernatant was collected. The collected supernatant was mixed with an equal volume of phenol: chloroform: isoamylalcohol (25:24:1 v/v) solution. All contents were agitated slowly for 30 min and centrifuged for 10 min at 10000 rpm to collect the supernatant. This step was repeated each with equal volume of chloroform:isoamylalcohol solution (24:1 v/v), added mixed again and the contents centrifuged to collect the supernatant. The DNA in supernatant was precipitated by adding 1/6 volume of isopropanol to the final supernatant and incubated for 1 h at room temperature. Then the mixture was centrifuged for 20 min at 10000 rpm, and the precipitated DNA pellet was washed with 70% ethanol. Finally, pellet was dissolved in 50 µl of sterile $T_{10}E_1$ buffer and stored at -20 ºC until further use.

3.3 Extraction of DNA from individual ingredient of panchagavya

3.3.1 Isolation of DNA from curd

Curd samples prepared in the laboratory using cow's milk were taken for DNA isolation. Extraction of DNA was done following the protocol given by Massana *et al.* (1997) with minor modifications. Fifteen ml of curd sample was homogenized into 30 ml phosphate buffer saline (PBS) containing (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 per litre, pH 7.4) and centrifuged at 13000 rpm for 10 minutes.

After centrifugation the supernatant was discarded and the pellet collected. The pellet was washed once with PBS buffer and resuspended in 1 ml Tris EDTA (TE) buffer containing (10 mM/l Tris-Cl pH 7.4, 1 mM/l EDTA). Lysozyme was added to achieve a final concentration of 10 mg/ml, and the mixture was incubated at 37 ºC for 30 min. To this mixture equal volume of CTAB buffer having composition (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl) was added and incubated at 65 ºC for 30 min. Then proteinase K (1 mg/ml) and 1 ml of (1% SDS) was added and incubated at 56ºC for 1 h. Then equal volumes of phenol:chloroform: isoamyl alcohol (25:24:1 v/v) were added and centrifuged at 13000 rpm for 10 min at 4 ºC to remove digested proteins. The supernatant was collected, and pooled with repeated steps under similar condition with equal volume of chloroform isoamyl alcohol (24:1 v/v). The upper phase was collected and equal volume of isopropyl alcohol was added and kept overnight at -20 ºC to precipitate DNA. After overnight incubation samples were centrifuged at 10000 rpm for 10 min at 4 ºC. The DNA pellet was dried and dissolved in 20 µl of sterile $T_{10}E_1$ buffer and stored at -20 ºC. The presence of DNA in the extracted product was checked on 1% agarose gel.

3.3.2 Isolation of DNA from milk

The total DNA from milk samples was extracted according to the method of Lopez *et al.* (2004). At the beginning, 0.5 ml of normal saline solution was added to one ml of the milk samples and was mixed by inverting vigorously for 10 times. Initially, these tubes were centrifuged at 13,000 rpm for 10 min at 4 $^{\circ}$C. The resultant supernatant was in clear form with cream pad in top layer. Both the pad and the supernatant were carefully removed, and the pellet left at the bottom of the tube was resuspended in one ml of normal saline solution and the mixture was centrifuged at 13000 rpm. The pellet, which contained microbial cells, was collected and dissolved in 860 µl of TES buffer containing [(50 mM Tris-HCl, 10 mM EDTA and 1% SDS; pH 8.0), 100 µl of 5 M guanidine hydrochloride, and 40 µl of proteinase K (20 mg/ml)]. The mixture was incubated in a water bath at 50 ºC overnight, and was left to cool at room temperature. The cellular debris and protein were removed by adding 500 µl Tris saturated phenol, and then inverting them 10 times, before centrifugation at 13000 rpm for 5 min at 4 ºC. The clear aqueous supernatant obtained was carefully

transferred to a new eppendorf tube. The DNA was precipitated by adding 1/6th volume of isopropanol to the final supernatant with 0.1 volumes of 3M sodium acetate (pH 5.2) as an antifoaming agent. The DNA pellet was obtained by centrifugation at 13000 rpm, for 10 min at 4 ºC. The DNA pellet was air dried, dissolved in 20 µl of sterile distilled water and preserved at -20 °C until further use.

3.3.3 Isolation of DNA from cow dung

Protocol used for isolating DNA from dung was similar to that mentioned for isolating DNA from panchagavya with slight variation in concentration of DNA extraction buffer components and incubation periods.

Metagenomic DNA was extracted with some modifications to procedures outlined by Kim *et al*., (2010); Kwon *et al.,* (2010). Five gram of freshly collected cow dung was added to 15 ml of the DNA extraction buffer, composition [100 mM Tris HCl (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM $CaCl_2$, 100 mM sodium phosphate buffer (pH 8.0), 1.5 M NaCl, 1.5% hexadecylmethylammonium bromide (CTAB)] and incubated for 1 h at 37 ºC with rotary agitation at 120 rpm. To the content 100 µl of Proteinase K (20 mg/ml) was added into the filtrate and incubated for 1 h at 37 ºC under shaking at 150 rpm. To this mixture 1.5 ml of 20% SDS solution was added and incubated at 65 ºC for 2 h in a water bath with intermittent mixing at every 20 min. Further, the contents were centrifuged for 10 min at 10000 rpm and the supernatant was collected. The collected supernatant was mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) solution, contents were agitated slowly for 30 min and centrifuged for 10 min, at 10000 rpm to collect the supernatant. This step was repeated and equal volume of chloroform:isoamylalcohol solution (24:1 v/v), was added again; the contents centrifuged to collect the supernatant. The DNA in supernatant was precipitated by adding 1/6th volume of isopropanol to the final supernatant and incubated for 1/2 h at room temperature. Then the mixture was centrifuged for 20 min, at 10000 rpm, and the precipitated DNA pellet was washed with 70% ethanol. Finally, pellet was dissolved in 100 µl of sterile $T_{10}E_1$ buffer until further use.

3.4 Microbial changes in phylloplane of soybean plants in response to application of panchagavya

3.4.1 Preparation of pots

Red sandy loamy soil was collected from Main Agricultural Research Station (MARS), UAS, Dharwad. The soil was sieved through 0.4 mm mesh. Further, it was autoclaved and three kg sterilized soil was filled into plastic pots size of 15 cm diameter.

3.4.2 Sterilization and pre-germination of soybean seeds

The seeds of soybean variety JS-335 used in the study were obtained from the All India Co-ordinate Research Project on Soybean, Dharwad, Centre, UAS Dharwad. Good quality soybean seeds, undamaged, uniform coloured and sized seeds were selected. The seeds were surface sterilized by immersing them in 0.1% of mercuric chloride solution for 1 min. Then seeds were rinsed with changes of sterile distilled water to remove traces of $HgCl_2$. Surface sterilized seeds were then soaked in sterile distilled water for one hour and such 10 seeds were transferred aseptically with sterile forceps on 0.8% water agar. The plates containing seeds were incubated in dark condition at 28 ºC for 24-36 h to enable seed germination. Five good, pregerminated seeds were sown in each pot and kept in green house. The moisture level in pots was maintained near field capacity throughout the study period.

3.4.3 Extraction of DNA from phylloplane

A pot culture experiment was conducted in the green house to assess the microbial changes in phylloplane of plant in response to phylloplane application of panchagavya. This experiment was carried out in four sets of pots each set replicated four times. First set of four pots were sprayed with 3 per cent panchagavya at 15^{th}, the second set at 30^{th}, the third set at both 15^{th} and 30^{th} days after sowing and the fourth set was kept as control without spraying. Leaf samples from all the sets including control were collected separately at 24 h, 48 h and 72 h after each spraying and metagenome from phylloplane was isolated following the method outlined by Delmotte *et al*. (2009).

Fifty fully developed leaves from each set of soybean plants were collected at each stage and placed in sterile polyethylene bag and immediately stored at 4ºC. Collected leaf samples were gently rinsed with sterile distilled water to remove dust particles adhering to leaf surface. Total microbial community DNA extraction from phylloplane was done using combination of different protocols given by Delmotte *et al*. (2009); Knief *et al*. (2008) and Kim *et al*. (2010). Out of fifty leaves ten leaves were placed in each polyethylene tube (Tarsons) containing 10 ml of $T_{10}E_1$ buffer and kept for gentle shaking at 60 rpm for 30 min. After shaking the leaves were removed and the buffer used for washing was taken in another new polyethylene tube. This mixture was centrifuged at 18,000 rpm for 15 min, the supernatant was discarded and the pellet was collected in 2 ml Eppendorf tube. To this one ml of DNA extraction buffer 100 mM Tris- HCl (pH-8), 100 mM Sodium phosphate buffer (pH-8), 100 mM EDTA (pH-8) and 1.5 M NaCl along with 50 µl of 10% SDS, were added and mixed. The contents were mixed properly by inverting for 6-8 times and then the sample was agitated at 1400 rpm at 60 °C for 30 min. Inverted tubes were centrifuged at 13200 rpm for 10 min at 4 ºC. The clear supernatant was collected and transferred to a fresh 2 ml micro-centrifuge tube. Purification of DNA in it was done by using organic solvents. Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added to the supernatant, to remove protein and other impurities. This homogenous mixture was thoroughly mixed and centrifuged at 13000 rpm for 20 min at room temperature. The clear supernatant was collected and transferred to a fresh 1.5 ml micro centrifuge tube. To this supernatant, equal volume of ice-chilled isopropanol was added for precipitating DNA. The mixture obtained were kept at -20 °C for overnight and then centrifuged at 13200 rpm for 10 min at 4 °C to discard the supernatant. The pellet thus obtained was washed with 70% ethanol, air-dried to remove the traces of ethanol and was dissolved in $T_{10}E_1$ buffer until further use. The concentration of extracted DNA was quantified using NanoDrop ND 1000 Spectrophotometer.

3.5 PCR Amplification of 16S rRNA gene using universal primers

Purified DNA samples were diluted using sterile distilled water in the ratio of 1:5 and used as template for carrying out PCR. The16S rDNA primer pair as detailed in (Table 1) was known to target different partial variable domains of 16S rRNA gene fragment which were used in the diversity analysis. The reaction mixture was

Table 1. Primers set used for PCR amplification of targeted region of 16S rRNA genes in the Metagenome

Primer pairs used		Primer Sequences	Expected product size (bp)	References
16S rDNA	PRBA 338 (Forward)	**5′-ACTCCTACG GGAGGCAGCAG- 3’	240 bp (V3)	Weisburg *et al.*(1991)
	PRUN 518 (Reverse)	5’-ATT ACC GCG GCTGCT GG- 3’		Muyzer *et al.* (1993)
	A905 (Forward)	**5’-TGAAACTTAAAGGAA-3’	154 bp (V5)	Yong and Pei (2009)
	A1059 (Reverse)	5’-GAGGWGGTGCATGGC-3’		Yong and Pei (2009)
	E-783 (Forward)	**5’-CAGGATTAGATACCC-3’	143 bp (V6)	Wang and Qian (2009)
	E-926 (Reverse)	5’ACTCAAAKGAATTGACGG-3’		Yong *et al.* (2009)
16S rDNA used for sequencing	V3 (Forward)	CCTACGGGNGGCWGCAG	460 bp	Xcelris NGS Bioinformatics Lab
	V4 (Reverse)	GACTACHVGGGTATCTAATCC		
** GC clamp added to the 5’ end of the primer 5’-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3’ *** GC clamp- 5’-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C- 3’				

Table 2. Programme for PCR amplification using different primers

Programme conditions	Initial denaturation	Denaturation	Primer Annealing				Extension	No. of cycles	Final extension	Hold
			PRBA338-PRUN518	A905-A1059	E783-E926	NSI1-Gcfung				
Temperature (ºC)	95 ºC	94 ºC	57.4 ºC	48.1ºC	48.3ºC	55 ºC	72 ºC	35	72 ºC	4 ºC
Time	7 min	30 seconds	45 seconds				45 seconds		10 min	

prepared for final volume of 10 µl which contained 0.25 pmol each of forward and reverse primers, 0.1 mM each of dNTP's, 1X Taq buffer A containing 1.5 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (GeNei, India). The PCR was performed in automated thermal cycler (Eppendorf master cycler, Germany) with following PCR programme, initial denaturation of seven minutes at 95 °C followed by denaturation at 94 ºC for 45 seconds, annealing at 55 ºC for 45 seconds and 45 seconds primer extension for 32 cycles, followed by 10 minutes final extension at 72 ºC. After completion of PCR, amplified products were analysed using 1% agarose gel. The details of primers their targeted regions are presented in table 1 while the PCR reactions are tabulated in Table 2.

3.6 Denaturing Gradient Gel Electrophoresis (DGGE) analysis

DGGE analysis was done for five samples of panchagavya collected at weekly interval, three samples of ingredients of panchagavya namely dung, curd and also milk and also phylloplane samples with and without panchagavya spray collected at regular intervals. PCR reaction was carried out on the template DNA of all the five samples of panchagavya taken at respective intervals, apart from the three metagenome samples of individual ingredient of panchagavya dung, curd, milk along with phylloplane metagenomic sample of panchagavya spray at different time intervals and their respective controls. The PCR product with an expected size of (240 bp, 143 bp, 154 bp and 450 bp for PRBA338-PRUN518, E-783-926, A-905-1059 and NS1-GCfung) expected were subjected to DGGE analysis by following protocol of Muyzer, (1999). Acrylamide gel gradient of 8-14% was used for separation of bands in the gel. The denaturing gradient maintained using gradient maker was 30-80%. The polyacrylamide gels were prepared with denaturing gradient from 30-80%, where 100% denaturant contained 7M urea and 40% formamide. DGGE was performed with Ingeny Phor U-2 system (Leiden, The Netherlands). After the denaturant gel was set completely (3 hr), a 5% staking gel was prepared and slowly poured over the denaturant gel avoiding the formation of air bubbles or gaps in between the two gels. Once the staking gel was set, the comb was removed and the whole cassette was placed into a preheated tank buffer containing 1X Tris-Acetate EDTA buffer (TAE buffer, pH-8). The PCR products were mixed with 1 µl of loading

dye and loaded onto the staking gel of the DGGE in aliquots of 20 µl per lane. Once the loading dye migrated to a length of staking gel, the tank buffer was kept in a circulating motion. The electrophoresis was performed at a constant voltage of 150 V for 18 h at 60 ºC in 1X TAE buffer. After complete run, gels were carefully removed from the unit and transferred to the OHP sheets. Gels were stained by using silver staining protocol as given by (Torsvik and Ovreas, 2002) using the following four solutions (Appendix III). Fixer solution to fix the bands on gel shakes slowly for 5 minute then given water wash for 1 min, then removed gel and placed in Impregnation solution for 5 minute with slight shaking then again given a water wash for 2 minute. Developer solution were added onto gel and shaken slowly for another 10 minutes when bands gets clearly visible onto gel shaking were stopped and gel carefully removed and kept in fixer solution for another 5 minute with slow shaking.

After staining, the gel was dried sufficiently and was analyzed using SynGene Gene Tools. Bands were scored in the Gene Tools, by giving lowest score to the least intense band in the gel. On the basis of this scored data, Sorenson's similarity coefficient, Shannon's diversity index and Range weighted richness, Pielou's evenness index, Pareto Lorenz evenness curve, Moving window analysis were calculated to analyze the similarity, diversity and carrying capacity, species evenness, functional organization of species, and microbial dynamics in the panchagavya and phylloplane microbial population.

3.6.1 Sorenson's similarity index (Cs):

Similarity in species composition between two samples was determined using this parameter. The overall similarity was calculated as the average of the pair-wise similarities.

$$C_s = \frac{2S\ (A \times B)}{N_A + N_B}$$

Where,

S (A x B) is the number of similar bands in sample A and sample B,

N_A and N_B are the total number of bands in sample A and sample B.

3.6.2 Shannon-Weaver index (H)

The diversity of taxa present in each sample was determined using this index (Shannon, 1948), which was calculated using the formula.

$$H = \sum_{i=1}^{S} (\mathbf{pi})[\mathbf{loge}(\mathbf{pi})]$$

Where,

S is the number of operational taxonomic units (OTUs) in one sample.

pi is the proportion of that OTU in the sample.

3.6.3 Range weighted richness (Rr)

It was measured on the basis of distribution of OTUs along the range of denaturing gradient of a DGGE profile. It is an estimate of the carrying capacity of the system and was calculated using the formula (Massimo *et al*., 2008).

$$Rr = (N^2 \times Dg)$$

Where,

N is the total number of bands in the pattern,

Dg is the denaturing gradient comprised between the first and the last band of the pattern.

3.6.4 Pielous eveness index (P):

Distribution of bacterial species throughout the stages of panchagavya was calculated as Pielou's evenness index using following formula;

$$P = H/H_{max}$$

Where,

H = Shannon diversity index

H_{max} = Lan (ln) of sample numbers

3.6.5 Pareto Lorenz (PL) evenness curve

In order to graphically represent the structure of a bacterial community (species distribution), Pareto–Lorenz (PL) evenness curves have been constructed based on the DGGE profiles generated by Bacterial, Archaeal and Eubacterial specific primers mentioned in Table1. For each DGGE lane, the respective bands were ranked from high to low, based on their intensities. Subsequently, the cumulative normalized numbers of bands were used on the X-axis, and their respective cumulative normalized intensities represented on the Y-axis.

3.6.6 Moving window analysis

Based on moving window analysis, the rate of change (D*t*) of parameter was calculated. With DGGE gel processing software a matrix of similarities for the densiometric curves of the DGGE patterns was calculated based on the formula:

Percent change = 100 – percent similarity

The percent change value matrix is used to perform moving window analysis by plotting the values between consecutive sampling points. Consequently, the rate of change (D*t*) value can be calculated as the average of the respective moving window curve data points. Higher the changes between the DGGE profiles of two consecutive sampling points, higher is the corresponding moving window curve data point and hence, higher the D*t* values.

3.7 Construction 16S rDNA metagenomic library

The metagenomics library preparation and sequencing of the amplicons were done using Ion Torrent Semiconductor Sequencing Methodology (Rothberg *et al.,* 2011). Samples were outsourced to Xcelris Company laboratory at Ahmadabad Gujarat for metagenomic library preparation and pyrosequencing. The procedure followed by the company is compiled below under three important steps.

3.7.1 Qualitative and quantitative analysis of Genomic DNA for metagenomic library construction

Quality of metagenomic DNA in panchagavya, sampled on 15th, 21st and 30th day was checked on 1% agarose gel by loading 5 µl of genomic DNA from each sample in a separate well. The gel was run at 110 V for 30 min. Similarly, quantity was checked by taking 1 µl of respective metagenomic DNA sample and loading them in Nanodrop 8000 for determining ratio at A260/280. The DNA was quantified using Qubit dsDNA BR Assay kit (Thermo Fisher Scientific Inc.) as per protocol mentioned.

3.7.2 Preparation of libraries for 2 x 300 bp run chemistry

The amplicon libraries were prepared using Nextera XT Index Kit (Illumina Inc.) as per the 16S Metagenomic sequencing library preparation protocol (Xcelris NGS library protocol). Primers for the amplification of the V3-V4 hyper-variable region (Table-1) of 16S rDNA gene of Eubacteria and Archaea were designed in Xcelris NGS Bioinformatics Lab. These primers were synthesized in Xcelris PrimeX facility. The amplicons with the Illumina adaptors were amplified by using i5 and i7 primers that add multiplexing index sequences as well as common adapters required for cluster generation (P5 and P7) as per the standard protocol with Illumina Inc. The amplicon libraries were purified by 1X AMpureXP beads and checked on Agilent High Sensitivity (HS) chip on Bioanalyzer 2100 and quantified on fluorometer by Qubit dsDNA HS Assay kit (Life Technologies).

3.7.3 Cluster Generation and Sequencing

After obtaining the optimum concentration for the library and the mean peak size from Bio analyzer profiles, library was loaded onto MiSeq at appropriate concentration (10-20 pM) for cluster generation and sequencing. Paired-End sequencing allowed the template fragments to be sequenced in both the forward and reverse directions on MiSeq. The kit reagents were used for binding of samples to complementary adapter oligos on paired-end flow cell. The adapters were designed to allow selective cleavage of the forward strands after re-synthesis of the reverse strand during sequencing. The copied reverse strand was then used to sequence from the opposite end of the fragment.

3.7.4 Bioinformatics analysis through QIIME software

The QIIME bioinformatics software was used for 16S rDNA metagenome analysis. This QIIME is comprehensive software comprising of tools and algorithms such as FastTree for heuristic based maximum-likelihood phylogeny inference (Price *et al*., 2010), the RDP classifier for the assignment of taxonomic data using a naive bayesian classifier (Wang *et al*., 2007). This allowed advanced analysis in the field of microbial community ecology. The following steps were performed:

- Stitching the PE data into single end reads
- Picking Operational Taxonomic Units (OTUs) based on sequence similarity within the reads, and picking a representative sequence from each OTU.
- Assigning the OTU to a taxonomic identity using reference databases.
- Calculated diversity metrices for each sample and compare the types of communities, using the taxonomic assignments.
- Generating Principle Component Analysis (PCA) plots to visually depict the differences between the samples, and then dynamically work with these graphs to generate publication quality figures.

OTU-picking identified highly similar sequences across the samples and provided a platform for comparisons of community structure. All the sequences from all the samples were clustered into Operational Taxonomic Units (OTUs) based on their sequence similarity. The OTUs were clusters of sequences, frequently intended to represent some degree of taxonomic relatedness done using u-clust, each resulting cluster typically represented a genus. Since each OTU may be made up of many sequences, a representative sequence for that OTU for downstream analysis was picked. This representative sequence would be used for taxonomic identification of the OTU. Alignment of the sequences against the representative sequences was performed using PyNAST. It takes into account a minimum length of 150 and a minimum percent identity of 75.0 for alignment purpose. Alignment was followed by assigning the taxonomy to microbial lineages. This result with the different bar charts and pie chart figures at different levels, the graphs are presented under experimental results.

4. EXPERIMENTAL RESULTS

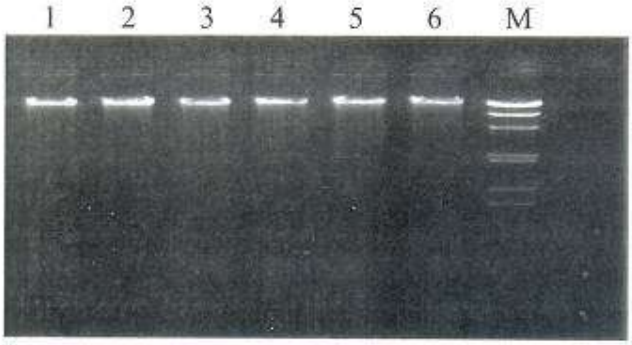
The present research mainly focused on studying the microbial succession that is likely to occur at different stages of panchagavya making mainly through culture independent approaches such as PCR-DGGE and amplicon sequencing. The DNA isolation protocol used in this study was a combination of different available protocols and was standardized step by step to develop one working protocol for achieving the set objectives. Accordingly, the PCR and DGGE conditions were also standardized for further analysis. Finally, Operational Taxonomic Units (OTUs) were sent for sequencing 16S rRNA gene segments amplified directly from isolated metagenomic DNA of panchagavya samples. This data was used to understand the complex microbiome structure in panchagavya. The results obtained during the study are presented in this chapter.

4.1 Isolation of metagenomic DNA from panchagavya

Total DNA was isolated at different stages of panchagavya, (after every 7 days starting from zero) using standardized protocol. The extracted DNA using this protocol was showing good quantity and optimum concentration with no visual shearing on 0.8% agarose gel electrophoresis. The average yield of DNA obtained was in the range of 1150- 1635 ng/µl with a purity ratio of 1.78-1.87 at 260/280 nanometre measured using Nano Drop ND 1000 Spectrophotometer (Plate 1).

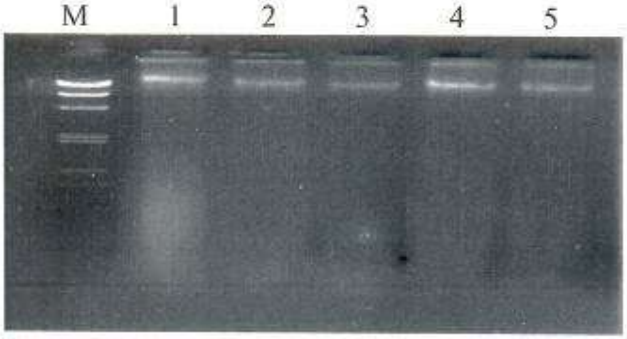
4.2 Isolation of metagenomic DNA from individual ingredient of panchagavya

Total metagenomic DNA from three individual ingredients of panchagavya namely; cow dung, curd, and cow milk were isolated using standardized protocols. The extracted DNA was showing optimum quality with no visual shearing. The average yield of DNA obtained from dung, curd, and milk ranged between 1550- 1820 ng/µl, 550- 920 ng/µl, and 350-580 ng/µl with purity ratio of 1.78-1.84, 1.74-1.88 and 1.72-1.79 respectively at 260/280 nm; measured using Nano Drop ND 1000 Spectrophotometer (Plate 2 to 4)



M- Marker *Mlu* single digest
1 – 1st day DNA sample
2 – 3rd day DNA sample
3 – 7th day DNA sample
4 – 14th day DNA sample
5 – 21st day DNA sample
6 – 30th day DNA sample

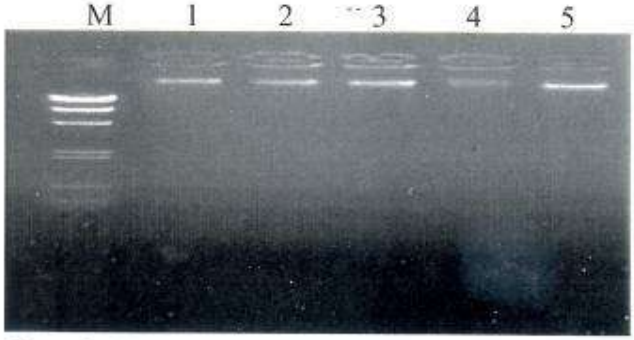
Plate 1: Metagenomic DNA isolated from different stages of panchagavya



M- *Mlu* single digest

1 to 5 – Milk DNA samples

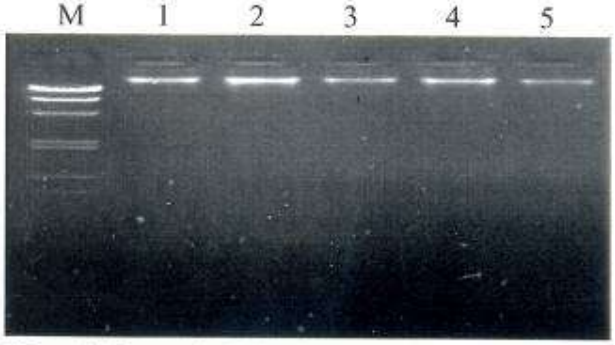
Plate 2: Metagenomic DNA isolated from cow milk



M- *Mlu* single digest

1 to 5 – Curd DNA samples

Plate 3: Metagenomic DNA isolated from curd



M- double digest *HindIII* and *EcoRI*

1 to 5 Dung DNA samples

Plate 4: Genomic DNA isolated from cow dung

4.3 Isolation of metagenomic DNA from Phylloplane of soybean

Metagenomic DNA was also extracted from phylloplane of soybean plant, receiving all the scheduled sprays of panchagavya at different time intervals and control plants. The metagenome showed good quality and quantity of DNA. Average concentration ranged from 800 to 1214 ng /µl (Plate 11).

4.4 PCR amplification of partial 16S gene using universal primers

Metagenomic DNA was isolated from different samples such as panchagavya at different stages, three individual ingredients and phylloplane of soybean. The DNA samples were subjected for PCR amplification, targeting variable region of partial 16S rRNA for bacteria. Primer pairs PRBA338-PRUN518, E-783-926 and A-905-1059 were used for targeting variable region V_3, V_5 and V_6 of 16S rRNA for eubacteria and archaea. All the primers used resulted in amplification of their targeted site with expected size 180 bp for PRBA338-PRUN518, 143 bp for E783-E926 and 154 bp for A905-A1059 respectively, indicating that primers targeted the exact region of 16S. There was no amplification in the negative control indicating no contamination in the PCR components (Plate 5 to 8).

4.5 DGGE analysis

Microbial diversity analysis of Panchagavya and phylloplane samples

PCR amplified product obtained using different primer pairs were separated on the basis of different migration profile on denaturing gradient gel. The banding patterns present in the DGGE profile of all the samples were scored using SynGene Tools. Further, numerical data were analyzed using different statistical tools for its diversity, richness, similarity, evenness, and dynamics using Shannon-weaver index (H), Range weighted richness (Rr), Sorenson's similarity indices, Pareto Lorenz curve and Moving window analysis respectively.

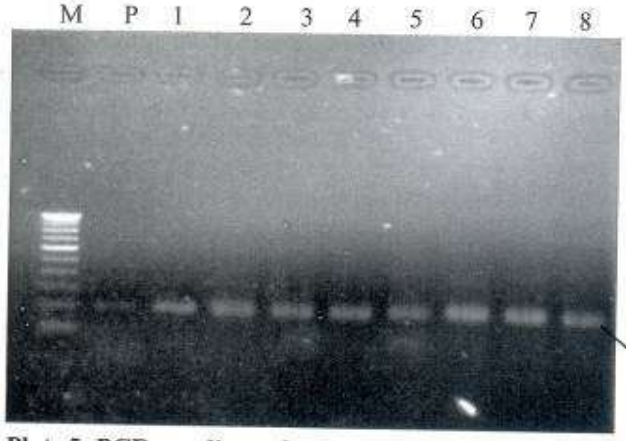


Plate 5: PCR amplicons from panchagavya metagenomic DNA using PRBA338-PRUN518 primer pair

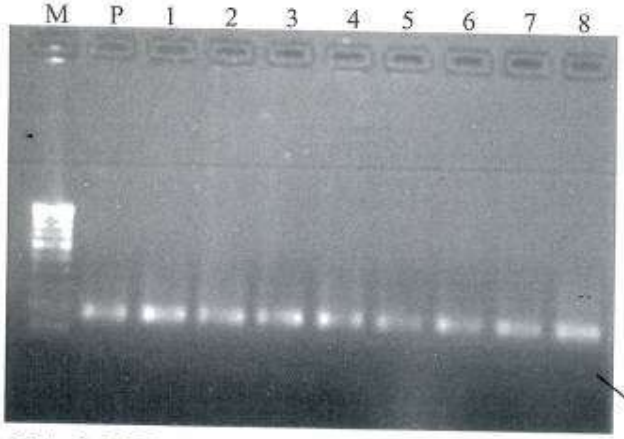


Plate 6: PCR amplicons from panchagavya metagenomic DNA using A905-A1059 primer pair

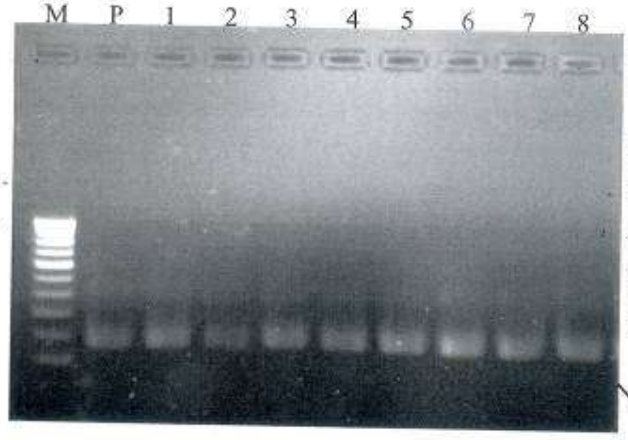


Plate 7: PCR amplicons from panchagavya metagenomic DNA using E783-E926primer pair

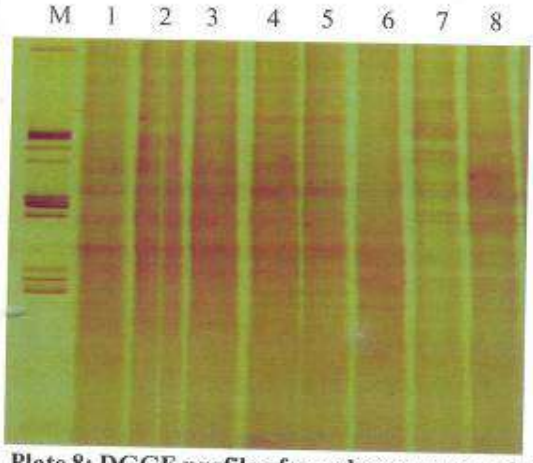


Plate 8: DGGE profile of panchagavya samples using PRBA338-PRUN518 primer pair

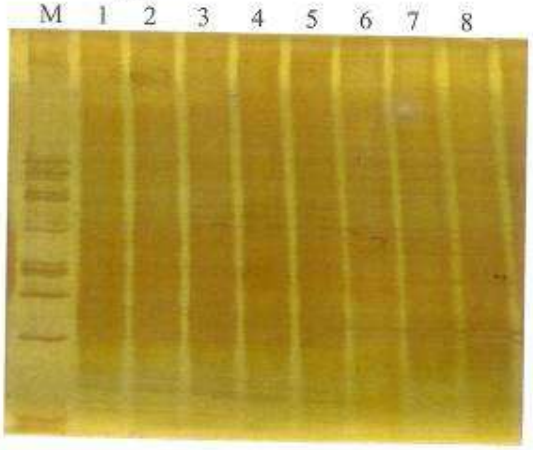


Plate 9: DGGE profile of panchagavya samples using A905-A1059 primer pair

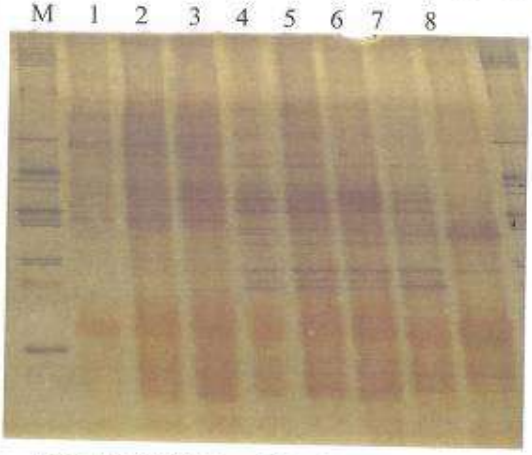


Plate 10: DGGE profile of panchagavya samples using E783-E926 primer pair

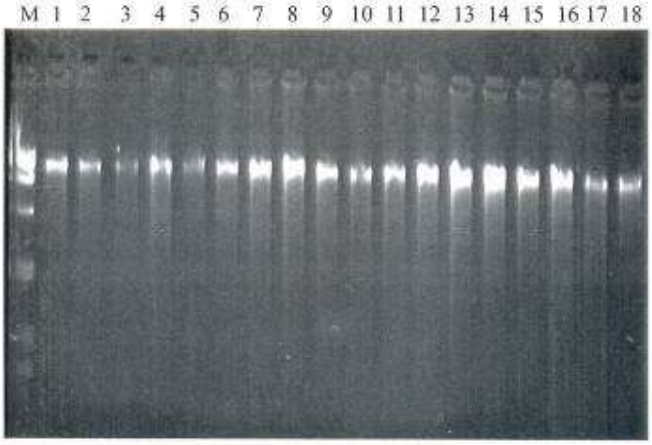


Plate 11: Metagenomic DNA isolated from phylloplane of soybean plant

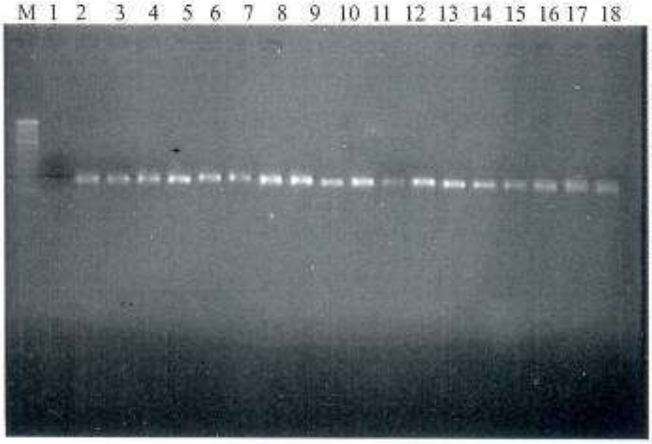


Plate 12: PCR amplicons from phylloplane metagenomic DNA using PRBA338-PRUN518 primer pair

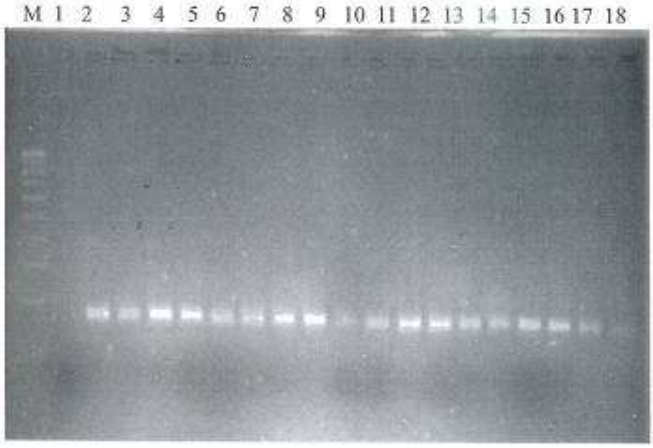


Plate 13: PCR amplicons from phylloplane metagenomic DNA using A905-A1059 primer pair

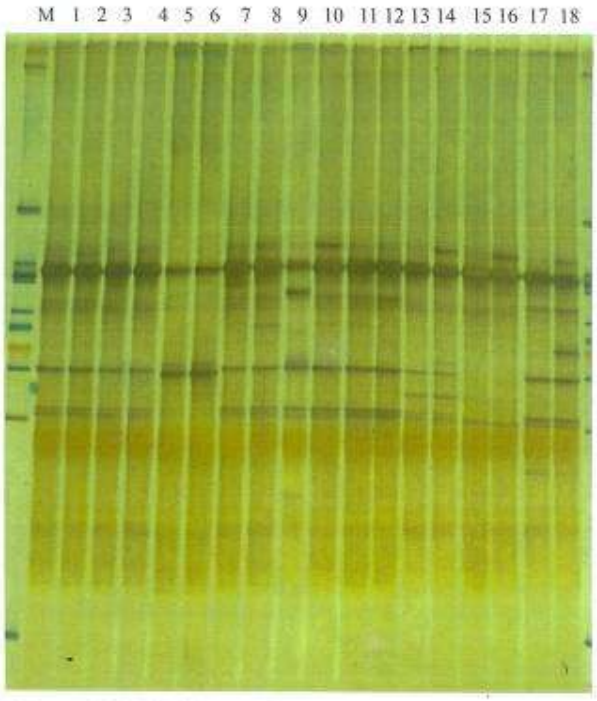


Plate 14: DGGE profile of phylloplane samples using PRBA338-PRUN518 primer pair

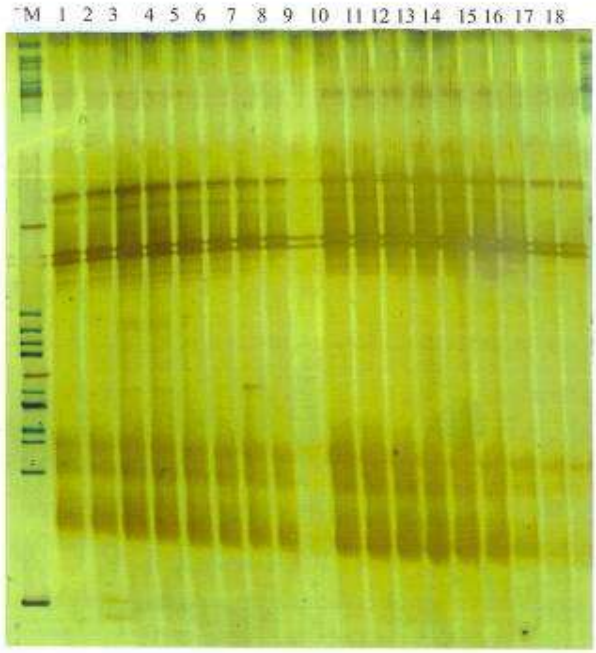


Plate 15: DGGE profile of phylloplane samples using A905-A1059 primer pair

4.5.1. Shannon diversity

Shannon diversity index is the most widely used parameter to access biodiversity although; it measures the average degree of distribution of species of a given individual within a randomly chosen population. The value of indices calculated for each randomly chosen sample helped to understand species richness and their evenness in the sample. When used for understanding microbial diversity it is likely to provide an insight into diverse group of microorganism and their relative distribution at given space and time.

Shannon diversity indices were calculated for the panchagavya under preparation on 3rd, 7th, 14th, 21st and 30th days of their preparation. Similarly, the index for each ingredient namely: fresh cow dung, fresh cow milk and fresh curd were calculated based on the DGGE profiles. It is evident from the values obtained (Table 3) for the three set of primers used that panchagavya at all the stages and individual fresh ingredients harbour a rich diversity of both eubacteria and archaeabacteria. It is interesting to know that the diversity of bacteria increased from 3rd day (2.79) to 14th day (2.96) during the process of panchagavya making there after declined on 21st day (2.82) to 30th day (2.35). This trend was also seen with eubacteria. However, the archaea bacterial diversity was found to decline on 14th day (2.73) from 7th day to 30th day (2.55). Among the ingredients the dung harboured the highest bacterial diversity including archaea bacteria. It is interesting to know that fresh samples of milk and curd did have a substantial diversity of archaeabacteria and eubacteria.

Overall the Shannon diversity indices reflected the higher diversity indices of 2.96 on 14th day of panchagavya sample and 3.31 of dung among the individual ingredients.

Shannon diversity index obtained from DGGE analysis of phylloplane microorganisms clearly indicated that spraying panchagavya increased microbial diversity in phylloplane at all stages 15th day (2.48) 15th + 30th day (2.68) and 30th day (2.59) against their respective unsprayed control samples. Similar trends

Table 3: Shannon diversity index of panchagavya samples

Primer pairs used	Stages of panchagavya					For individual ingredient		
	3rd Day	7th Day	14th Day	21st Day	30th Day	Dung	Milk	Curd
PRBA338-PRUN518	2.74	2.80	2.96	2.82	2.35	3.31	2.71	2.22
A905-1059	2.72	2.84	2.73	2.58	2.55	2.87	2.61	2.62
E783-926	2.43	2.44	2.91	2.79	2.68	2.19	2.54	1.85

Table 4: Shannon diversity index of phylloplane sample

Sampling interval						
Treatments	15th Day		15th + 30th Day		30th Day	
Primer pairs	Control	Sprayed	Control	Sprayed	Control	Sprayed
PRBA338-PRUN518	2.33	2.48	2.42	2.68	2.44	2.59
0A905-1059	1.94	2.23	2.12	2.49	2.11	2.38

were observed for both primer sets, one specific for total bacteria and the other archaeabacteria. The phylloplane diversity in control (unsprayed) plants also, increased with duration (1.94 on 15th day and 2.12 on 30th) day although these plants were sprayed only with water (Table 4).

4.5.2 Range weighted richness (Rr)

The values of Range-Weighted Richness (Rr) were estimated on a denaturing gradient of 30-70 per cent range (Table 5). Number of bands or OTUs under each sample were critically analysed to elucidate species richness in each respective sample. The range weighted richness for total bacterial, archaeal and eubacterial specific primer were found to be higher (all are more than 30) for all the panchagavya samples drawn at different intervals and for individual ingredients of panchagavya.

Similarly, the values of range weighted richness for microbial communities of phylloplane both control and sprayed at regular intervals were found to be higher (all are more than 30). However, the values for control were much lower than those observed with sprayed phylloplane irrespective of the time and number of spray (Table 6).

4.5.3 Sorenson's pair-wise similarity index

PCR-DGGE profile generated by using PRBA338-PRUN518, A905-A1059 and E783-E926 primer pairs were analyzed by using Sorenson's similarity index; a statistical tool to compare similarity in microbial community at different stages of panchagavya making and amongst the ingredients used in panchagavya making (Table 7). A similarity index of 100 per cent indicated that DGGE profiles were identical while completely different profiles result in a value of 0 per cent.

Sorenson's similarity index of panchagavya sample with PRBA338-PRUN518 primer pair showed that the sample drawn at 14th and 21st day shared the highest microbial similarity of 65 per cent followed by samples drawn at 3rd and 7th day with 55 per cent similarity. However, samples drawn on 14th and 30th day shared very low values of similarity 14 per cent. Similarity index of individual ingredients with that of the panchagavya at different stages showed very low

Table 5: Range weighted richness of panchagavya samples

Primer pairs	Stages of panchagavya					Individual ingredient		
	3rd Day	7th Day	14th Day	21st Day	30th Day	Dung	Milk	Curd
PRBA338-RUN518	209.58	228.92	181.88	114.79	122.11	556.87	64.8	74.8
A905-1059	434.6	327.6	329.7	381.4	141.1	421.2	235.8	100.8
E783-926	116.87	120	194	148	191.48	66	127.57	60.5
Rr < 10: low range weighed richness, Rr ≈10-30 medium range weighed richness and Rr > 30: high range weighed richness								

Table 6: Range weighted richness for microbial community analysis of phylloplane samples

Samples representing spray schedule						
Sampling interval	15th Day		15th + 30th Day		30th Day	
Primer pairs	Control	Sprayed	Control	Sprayed	Control	Sprayed
PRBA338 - PRUN518	98	125	108	144	111	136
A905-1059	114	132	111.3	144	115	138

values. Similarly, curd with 3rd day and 7th day samples of panchagavya and milk with 30th day samples of panchagavya showed very low similarity based on the position of their OTUs and their number. Curd and milk showed 13 per cent similarity, milk and dung showed 20 per cent similarity whereas, curd and dung showed 12 per cent similarity in microbial community.

Panchagavya at all the stages of preparation showed an overall 31 per cent similarity of total bacterial population. When all the stages of panchagavya were compared with dung, milk and curd it showed an overall similarity of 35 per cent, 21 per cent and 9 per cent respectively (Table 7).

PCR-DGGE profile generated by using archaeal specific primer pair: A905-A1059 was analyzed using Sorenson's similarity indices (Table 8). The results showed that panchagavya samples drawn on 14th and 21st day had the highest similarity, followed by the samples drawn on 30th day with milk, 7th day with dung, 21st day with curd. The lowest similarity was observed between 3rd and 30th day panchagavya sample. Among the individual ingredients used for making panchagavya, milk and curd shared the highest Sorenson's similarity index value (0.28 %) for archaeabacteria (Table 8).

Panchagavya at all the stages of preparation showed an overall 44 per cent similarity of total bacterial population. When all the stages of panchagavya were compared with dung, milk and curd it showed overall similarity of 34 per cent, 32 per cent and 34 per cent respectively.

The values of Sorenson's similarity index calculated using profile generated by DGGE analysis of PCR products using primer E783-E926 clearly suggested that the cow dung microflora shared the highest similarity of 58 per cent with sample drawn on 3rd day of panchagavya (Table 9). This was followed by similarity of microflora present in panchagavya sample drawn on 14th and 21st day (55 %). The similarity in microflora of milk and 30th day panchagavya sample was 51 per cent. The similarity index for rest of all samples was less than 50 per cent.

Table 7: Sorenson's pair-wise similarity index for microbial community of panchagavya sample with PRBA338-PRUN518 primer pair

	Dung	3rd Day	7th Day	14th Day	21st Day	30th Day	Milk	Curd
Dung	1.00							
3rd Day	0.43	1.00						
7th Day	0.42	0.55	1.00					
14th Day	0.33	0.17	0.28	1.00				
21st Day	0.33	0.24	0.36	0.65	1.00			
30th Day	0.28	0.21	0.23	0.14	0.15	1.00		
Milk	0.20	0.21	0.22	0.16	0.22	0.04	1.00	
Curd	0.12	0.05	0.05	0.11	0.10	0.02	0.13	1.00

Table 8: Sorenson's pair-wise similarity index for microbial community of panchagavya sample with A905-A1059 primer pair

	Dung	3rd Day	7th Day	14th Day	21st Day	30th Day	Milk	Curd
Dung	1.00							
3rd Day	0.38	1.00						
7th Day	0.40	0.36	1.00					
14th Day	0.38	0.32	0.38	1.00				
21st Day	0.28	0.21	0.32	0.52	1.00			
30th Day	0.26	0.20	0.24	0.32	0.25	1.00		
Milk	0.23	0.21	0.28	0.22	0.14	0.43	1.00	
Curd	0.25	0.25	0.28	0.24	0.43	0.28	0.28	1.00

Table 9: Sorenson's pair-wise similarity index for microbial community of panchagavya sample with E783-E926 primer pair

	Dung	**3rd Day**	**7th Day**	**14th Day**	**21th Day**	**30th Day**	**Milk**	**Curd**
Dung	1.00							
3rd Day	0.58	1.00						
7th Day	0.21	0.34	1.00					
14th Day	0.22	0.25	0.30	1.00				
21th Day	0.15	0.15	0.18	0.55	1.00			
30th Day	0.15	0.20	0.19	0.26	0.44	1.00		
Milk	0.18	0.18	0.20	0.23	0.40	0.51	1.00	
Curd	0.05	0.10	0.12	0.14	0.17	0.21	0.33	1.00

PCR-DGGE profile generated using eubacterial specific primer pair E783-E926 showed that the dung and panchagavya samples drawn on 3rd, 14th and 21st days and also between 21st and 30th day samples had higher similarity indices. This was followed by milk and 30th day. However, milk and 21st day sample curd and 21st sample day were also showing higher similarity indices. The Sorenson's similarity between curd and 3rd day and dung and curd were found to be very low (Table 9).

Panchagavya at all the stages of preparation showed an overall 31 per cent total bacterial similarity. When all the stages of panchagavya were compared with dung, milk and curd overall similarity of 26 per cent, 30 per cent and 14 per cent respectively was observed.

4.5.4 Pielou's evenness index

Pielou's evenness index was calculated for the metagenome isolated from five different samples of panchagavya and three ingredients of panchagavya using three primer pairs listed in table 10. The population of bacteria was found to be highly even in most of the samples except for the total bacterial population in the panchagavya sample drawn on 30th day, archaeabacterial population in panchagavya samples drawn on 21st day and archaeal population in milk and eubacterial population in dung. The highest evenness was found in total bacteria of cow milk (0.91) followed by panchagavya sample drawn on 21st day (0.88). The values for bacterial evenness in rest of the samples were between (0.74 - 0.88).

The Pielou's evenness index of bacterial population in phylloplane (Table 11) appeared to be much lower than those observed in panchagavya and their ingredients. It is interesting to note that the population of bacteria was moderately even in the phylloplane unsprayed with panchagavya on 15th day control (0.71) and that sprayed on 15th day (0.70), 30th day control (0.71) and 30th day sprayed (0.74). While, archaeabacterial population was found to be highly even irrespective of sprays both in panchagavya sprayed on 15th day (0.82) and unsprayed samples of 30th day (0.82). Archaeabacterial population showed high even in all the phylloplane samples except those which didn't receive spray on 15th day (0.73) and those with sprayed on 30th day (0.79).

Table 10: Pielou's evenness index of panchagavya sample drawn at different stages

Primer pairs	Stages of panchagavya					Individual ingredient		
	3rd Day	7th Day	14th Day	21st Day	30th Day	Dung	Milk	Curd
PRBA338-PRUN518	0.84	0.84	0.88	0.89	0.74	0.88	0.91	0.75
A905-1059	0.75	0.81	0.79	0.73	0.83	0.79	0.74	0.76
E783-926	0.75	0.76	0.84	0.82	0.78	0.74	0.77	0.60

Table 11: Pielou's evenness index of phylloplane samples

Sampling interval / Primer pairs	Stages of spraying Panchagavya					
	15th Day		15th + 30th Day		30th Day	
	Control	Sprayed	Control	Sprayed	Control	Sprayed
PRBA338-PRUN518	0.71	0.70	0.78	0.76	0.71	0.74
A905-1059	0.73	0.82	0.82	0.84	0.86	0.79

Pielou's evenness index value 1 indicated that species distribution in sample was highly even while, an index value of 0 indicated highly uneven distribution of species present in sample.

4.5.5 Functional organization (Fo)

Pareto Lorenz (PL) curves were derived based on DGGE pattern obtained using three distinct primer pairs namely; PRBA338-PRUN518, A905-1059 and E783-E926 for panchagavya samples and two primer pairs PRBA338-PRUN518 and A905-1059 for phylloplane. PL curve of panchagavya sample using PRBA338-PRUN518 primer pair showed that more than 80 per cent of population of bacteria comprises of about 20% of species which are functionally more organized (Fig. 1)

PL curve for same set of genome using archaea specific primer pair (Fig. 2) reflect functionally highly organized population of archaea bacteria in panchagavya and their ingredients. As more than 80 per cent of functionally related archaeabacteria come from less than 20 per cent of archaea bacterial species.

Similar trend was also followed for the eubacterial functional organization with eubacteria with eubacterial specific primer pair (Fig. 3).

PL curve obtained from the DGGE pattern of phylloplane metagenome using PRBA338-PRUN518 primer (Fig. 4) and A905-A1059 primer (Fig.5) also showed that both the bacterial groups in the phylloplane contain functionally highly related bacteria. In all these curves (Fig. 1 to 5) the values appeared to be represented by less than 20 per cent of bacterial communities which are functionally dominant in panchagavya samples at different stages of its making.

Curves with 25 per cent PL range represents the community with high evenness, while those with 45 per cent PL range and the area around it represent communities with lower evenness as compared to the 25 per cent PL curve. Curve with the 80% PL range value, represents a specialized community in which a small amount of the species are dominant and all the others are present in low numbers. In this study PL curves showed a value of more than 80%.

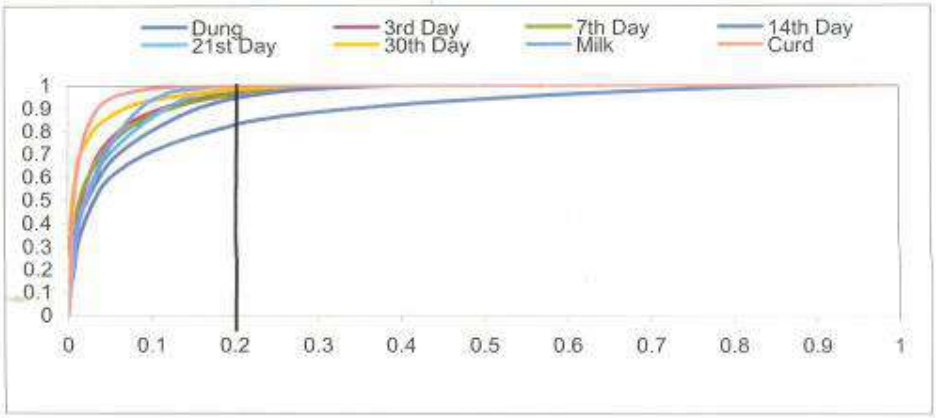


Fig. 1. Pareto Lorenz curve of panchagavya metagenome using PRBA338- PRUN518 primer pair

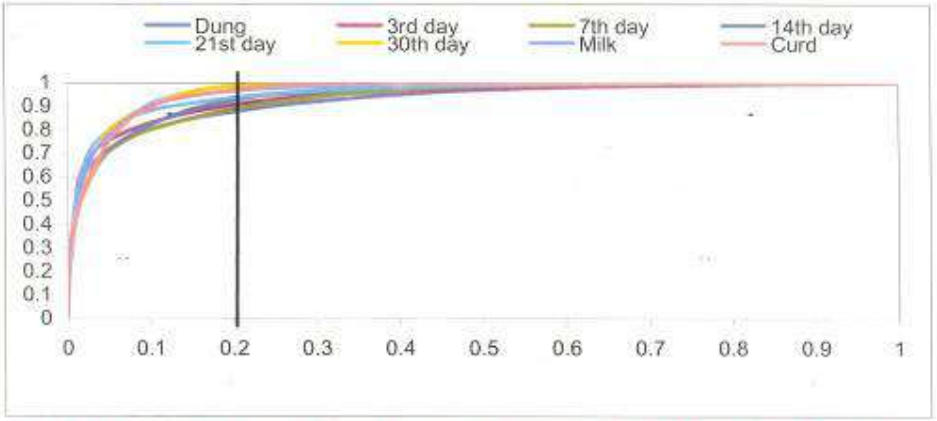


Fig. 2. Pareto Lorenz curve of panchagavya metagenome using A905-A1059 primer pair

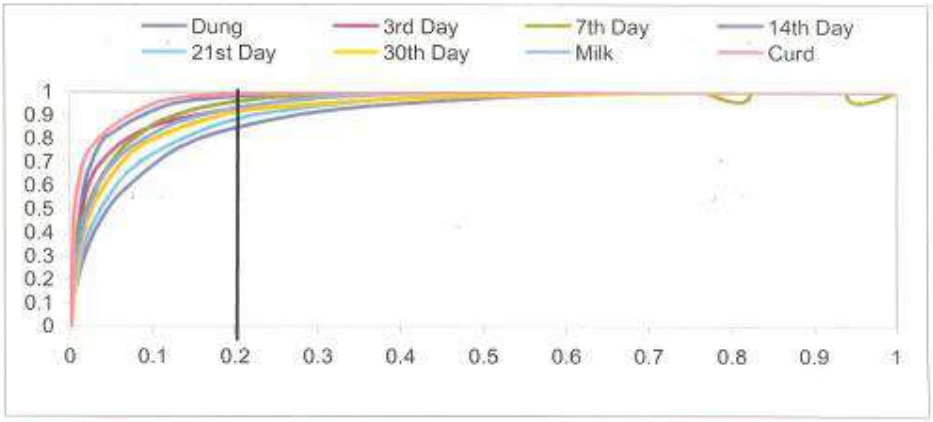


Fig. 3. Pareto Lorenz curve of panchagavya metagenome using E783-E926 primer pair

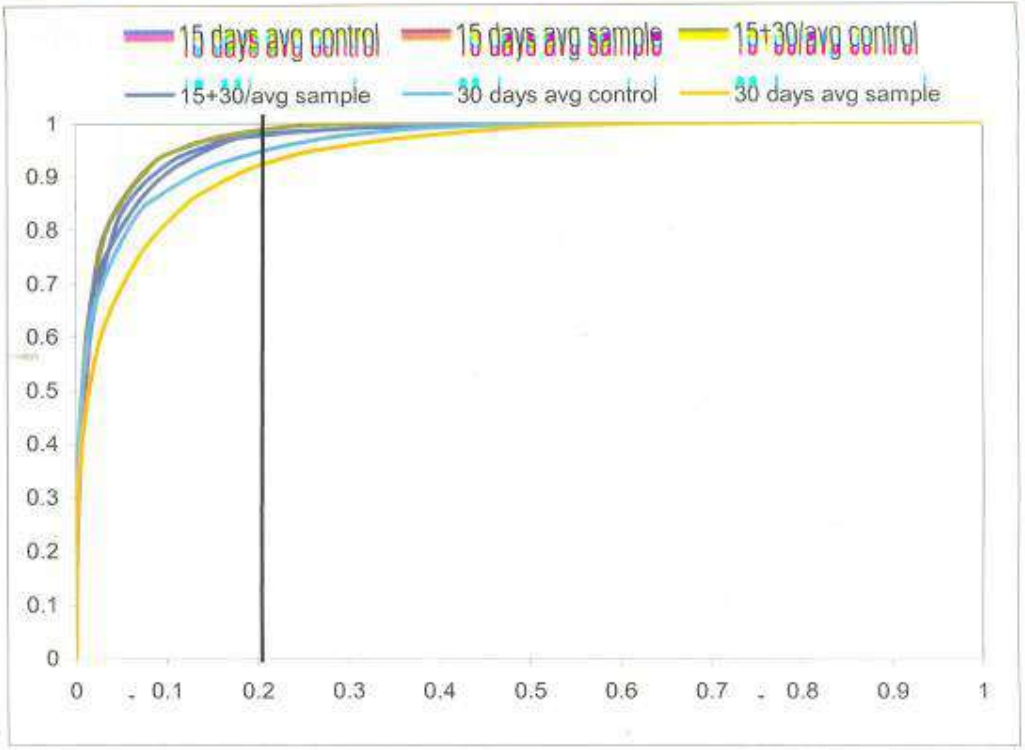


Fig. 4. Pareto Lorenz curve of phylloplane metagenome using PRBA338-PRUN518

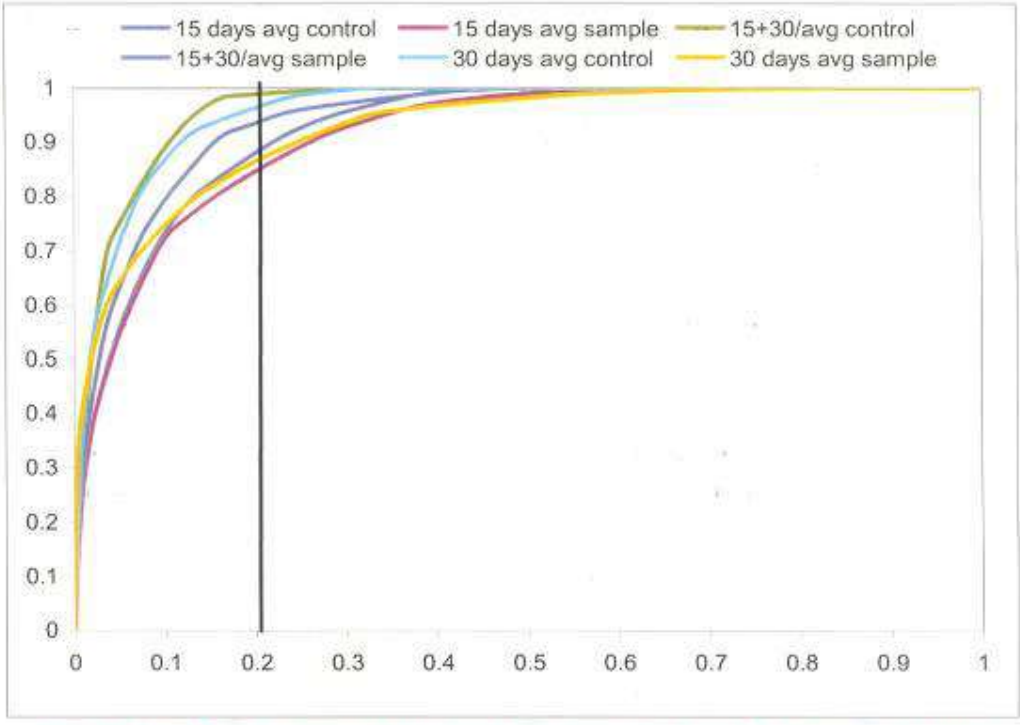


Fig. 5. Pareto Lorenz curve of phylloplane metagenome using A905-1059 primer pair

4.5.6 Microbial community dynamics

Microbial community dynamics is the average rate of change in parameter and the degree of change between consecutive DGGE profiles of the same community over a fixed time interval. In the present study bacterial community dynamics of the genomic DNA of panchagavya and phylloplane were studied using moving window analysis (Fig. 6 and 7). The average rate of changes in bacteria observed at five consecutive intervals of panchagavya using the three primer pairs namely; PRBA338-PRUN518, A905 - A1059 and E783-E926 were respectively 17.8, 8.51 and 10.90. Microbial community dynamics in panchagavya samples draw using PRBA338-PRUN518 primer pair drastically reduced from 56.5 to 45.45 in 7^{th} day sample again increased on 14^{th} day sample and almost a double increase was observed from 21^{st} day sample (34.74) to 30^{th} day sample (84.71) (Fig. 6).

Microbial community dynamic values for primer pair; A905-A1059 specific for archaebacteria, appeared to be steady till 14^{th} day (ranged in between 62.21 to 62.31), then drastically reduced on 21^{st} day (48.32) and again increased till the 30^{th} day (75.08) during panchagavya preparation.

Microbial community dynamics of eubacteria using E783-E926 primer pair showed interesting trends of community dynamics. There was a steady increase from 3^{rd} to 14^{th} day (from 42.27 to 69.92) and transiently dropping on 21^{st} day (45.0) and subsequently increasing to 55.91 on 30^{th} day metagenomic sample of panchagavya during the process of its preparation.

Microbial community dynamic in the metagenome of unsprayed and sprayed phylloplane samples was compared. The bacterial dynamics increased with spraying and was the highest with samples receiving sprays on 15^{th} and 30^{th} days. The phylloplane metagenomes from samples either unsprayed or sprayed on both 15^{th} and 30^{th} day showed higher bacterial dynamics with values of 41 and 65 respectively. These dynamics values were higher than those recorded with sprayed or unsprayed on 30^{th} day only. In general the bacterial dynamics in case of sprayed samples were higher than their unsprayed counterparts (Fig. 7). The change in the microbial communities due to spraying was 4.96 per cent and those in unsprayed was 11.08 per cent.

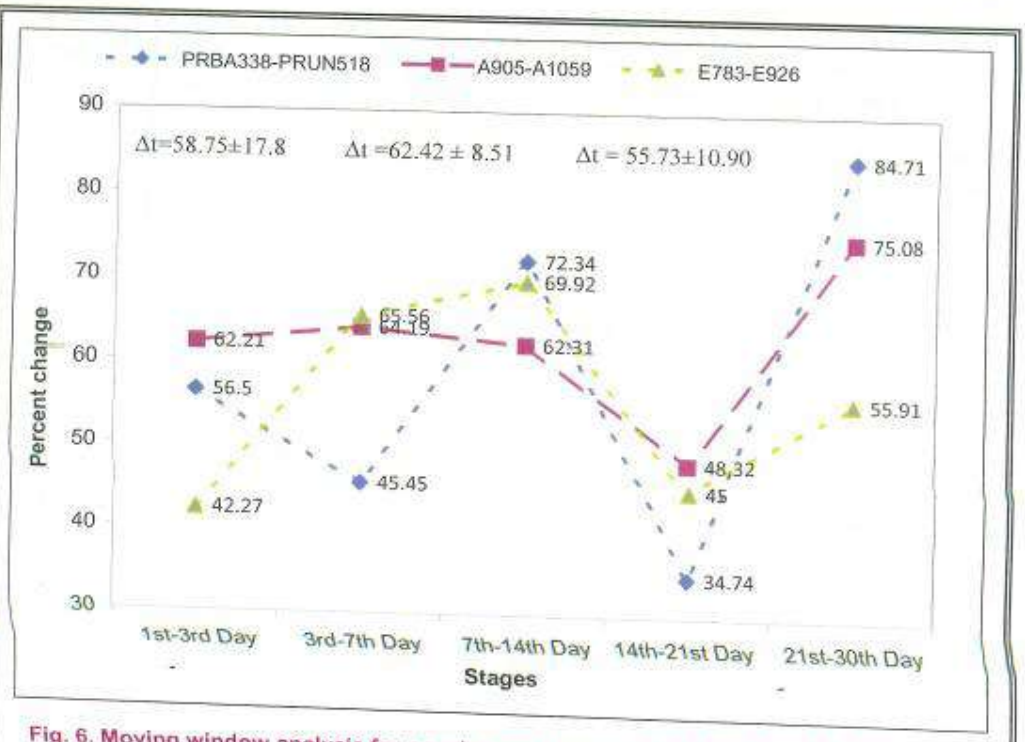


Fig. 6. Moving window analysis for panchagavya sample using PRBA338-PRUN518, A905-A1059, E783-E926 primer pair simultaneously

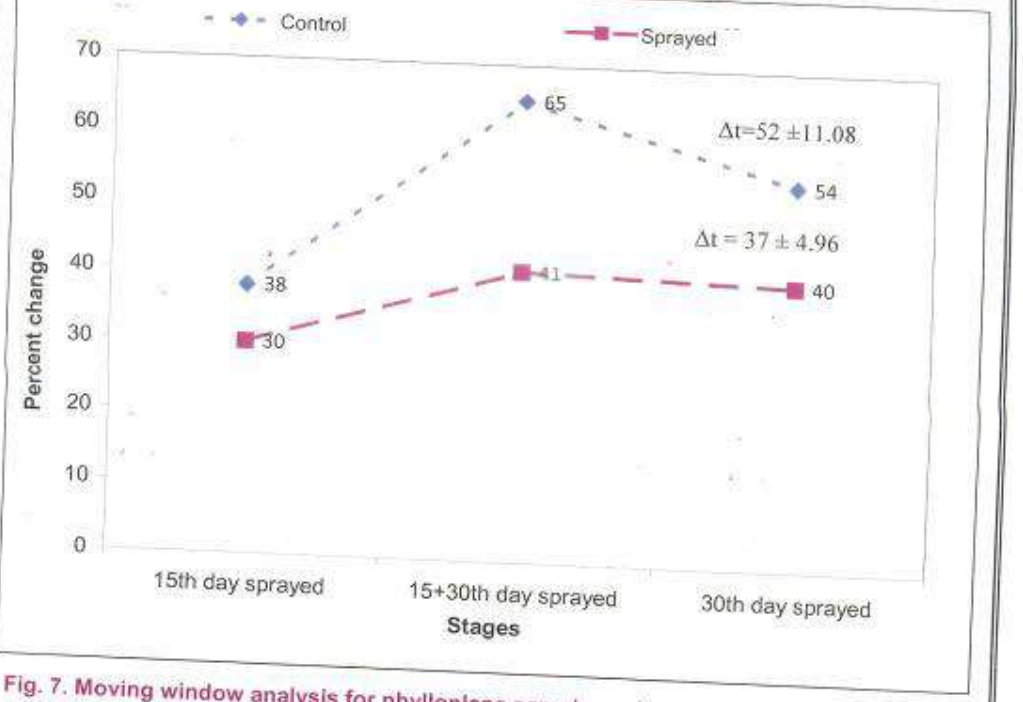


Fig. 7. Moving window analysis for phylloplane samples using PRBA338-PRUN518 primer pair simultaneously

Table 12: Gas Chromatography Mass Spectroscopy (GCMS) analysis of secondary metabolites present in panchagavya sample at different periods

Sl. No	Name of the compound	7th Day	14th Day	21st Day	30th Day
1	1,2-Diaminoethane, 1,2-bis 2-hydroxyphenyl	+	-	-	-
2	Cetene	+	+	+	
3	Methyl tetradecanoate	+	+	+	-
4	1-Nonadecene	+	-	-	-
5	Hexadecanoic acid, methyl ester	+	+	+	+
6	Tridecanoic acid, methyl ester	+	-	-	-
7	Pentadecanoic acid, methyl ester	+	+	+	+
8	Heptadecanoic acid, methyl ester	+	-	-	-
9	9-Octadecenoic acid , methyl ester	+	-	+	+
10	Octanoic acid	-	+	-	-
12	n-Decanoic acid	-	+	+	+
13	Dodecanoic acid, methyl ester	-	+	+	-
14	Methyl myristoleate	-	+	+	+
15	Undecanoic acid, methyl ester	-	+	+	+
16	Pentadecanoic acid, 14-methyl-, methyl ester	-	+	+	-
17	Tetradecanoic acid	-	+	+	-
18	9-Hexadecenoic acid, methyl ester,	-	+	+	+
19	Methyl 8-methyl-nonanoate	-	+	+	-
20	Pentanoic acid	-	-	+	+
21	Hexanoic acid	-	-	+	+
22	Octanoic acid, methyl ester	-	-	+	-
23	Benzeneacetic acid, methyl ester	-	-	+	+
24	Hexanoic acid, butyl ester	-	-	+	-
25	Octanoic acid	-	+	+	-
26	Benzoic acid	-	-	+	-
27	1,5-Hexadiyne	-	-	+	-
28	Dodecanoic acid	-	-	+	+
29	Oleic Acid	-	-	+	+
30	Methyl stearate	-	-	+	+
31	Ethyl Oleate	-	-	+	-
32	Hexadecanoic acid, butyl ester	-	-	+	-
33	Trans-13-Octadecenoic acid	-	-	+	-
34	Heneicosanoic acid, methyl ester	-	-	-	+
35	Butyl myristate	-	-	-	+
36	11-Dodecenoic acid, 10-hydroxy-, methyl ester	-	-	-	+

Table 13: Published functional attributes of the compounds identified from panchagavya using GCMS

Sl. No	Name of the compound	Molecular formula	MW	Purity (%)	RT	Reported biological activity	References
1	1,2-Diaminoethane, 1,2-bis(2-hydroxyphenyl)	$C_{14}H_{16}N_2O_2$	244.28	62 %	14.60	Not reported	
2	Cetene	$C_{16}H_{32}$	224.42	64 %	15.42	Not reported	
3	Methyl tetradecanoate	$C_{14}H_{28}O_2$	228.37	93%	17.17	Not reported	
4	1-Nonadecene	$C_{19}H_{38}$	266.50	99.9 %	17.94	Not reported	
5	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45		18.25	Antioxidant, Flavor, 5-Alpha reductase inhibitor, Pesticide, Nematicide, Hypocholesterolemic Pesticide Hypocholesterolemic	Sermakkani and Thangapandian (2012) and Vijayakumar and Pannerselvam (2013)
6	Tridecanoic acid, methyl ester	$C_{14}H_{28}O_2$	228.37	73%	19.30	Not reported	
7	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256.42	70 %	19.30	Antibacterial and Antifungal	Agoramoorthy *et al*. (2007)
8	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	284.47	59%	20.28	Antioxidant	Silva *et al*. (2013)
9	9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296.48	78%	19.11	Anti-inflammatory, Cancer preventive, InsectifugeAnti-acne, Hepatoprotective, Antihistamine, Nematicide, Antiandrogenic, Anti-eczemic, Anti-arthritic, Hypercholesterolemic, 5-Alpha reductase inhibitor,	Omotoso *et al.* (2014)
10	Octanoic acid	C8H16O2	144.21	69 %	11.23	Enhance natural flavour of fruit crops, antimicrobial food additive,	Khalaf *et al.* (2014)
12	n-Decanoic acid	$C_{10}H_{20}O_2$	172.26	73 %	13.33	Not reported	
13	Dodecanoic acid, methyl ester	$C_{11}H_{22}O_2$	186.29	66 %	14.85	Anti inflammatory effect, Antiprotozoal, and antimicrobial activity.	Selvin *et al*. (2009)
14	Methyl myristoleate	$C_{15}H_{28}O_2$	240.38	99 %	17.04	Not reported	
15	Undecanoic acid, methyl ester	$C_{12}H_{24}O_2$	200.31	71%		Not reported	
16	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	270.45	67 %	17.21	Antioxidant	Vijisaral and Arumugam (2014)
17	Tetradecanoic acid	$C_{14}H_{28}O_2$	228.37	91 %	18.33	Not reported	
18	9-Hexadecenoic acid, methyl ester,	$C_{17}H_{32}O_2$	268.43	72 %	19.10	Not reported	
19	Methyl 8-methyl-nonanoate	$C_{10}H_{20}O_2$	254.34	65 %	19.32	Not reported	
20	Pentanoic acid	$C_5H_{10}O_2$	102.13	78 %	7.71	Antioxidant detoxification, Antigastrinic, Cytoprotective activity	Steven *et al*. (2014)
21	Hexanoic acid	$C_6H_{12}O_2$	116.15	74 %	8.68	Antifungal, antibacterial, viral resistance, Antihyperglycemic, Enhance estrogenic and androgenic activity,	Alva *et al*. (2012)

22	Benzeneacetic acid, methyl ester	$C_9H_{10}O_2$	150.17	80 %	10.29	Not reported	
23	Hexanoic acid, butyl ester	$C_{10}H_{20}O_2$	172.26	77 %	10.42	Not reported	
24	Octanoic acid, Methyl ester	$C_9H_{18}O_2$	158.23	66 %	10.22	Insectisidal activity, antidepressant and antioxidant, prevent mitochondrial damage, Antiprolifirative activity	Li *et al*. (2013)
25	Benzoic acid	$C_7H_6O_2$	122.12	68 %	11.65	Antileishmania, Antibacterial Activities and Cytotoxicity against Macrophages.	Arshad *et al.* (2014)
26	1,5-Hexadiyne	C_6H_6	78.11	73 %	13.33	Not reported	
27	Dodecanoic acid	$C_{12}H_{24}O_2$	200.3	63 %	15.91	Not reported	
28	Oleic Acid	$C_{18}H_{34}O_2$	282.46	60 %	17.65	Cancer preventive, Dermatitigenic, Anemiagenic, Antiandrogenic, Insectifuge,	Vijisaral and Arumugam, (2014)
29	Methyl stearate	$C_{19}H_{38}O_2$	298.5	89 %	21.29	Not reported	
30	Ethyl Oleate	$C_{20}H_{38}O_2$	310.51	89 %	21.63	antifungal and antibacterial activity.	Govindaraju *et al*. (2014)
31	Hexadecanoic acid, butyl ester	$C_{20}H_{40}O_2$	312.53	69 %	21.79	Anti-tumor Activities,	Osman, *et al*. (2011)
32	Trans-13-Octadecenoic acid	$C_{18}H_{34}O_2$	282.86	83 %	23.30	antioxidant and antitumor properties	Khalaf *et al*. (2014)
33	Heneicosanoic acid, methyl ester	$C_{22}H_{44}O_2$	340.58	69 %	19.33	Not reported	
34	n-hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	68 %	22.21	Antioxidant, Hypercholesterolemic, Lubricant, Nematicide, Pesticide, Hemolytic 5-Alpha reductase inhibitor, Flavour, Antiandrogenic	Omotoso *et al*. (2014)
35	Butyl myristate	$C_{18}H_{36}O_2$	284.47	69 %	21.79	Not reported	
36	11-Dodecenoic acid, 10-hydroxy-, methyl ester	$C_{13}H_{24}O$	228.32	72 %	23.05	Not reported	

4.6 Gas chromatography and mass spectral (GCMS) analysis of metabolites

The extracted secondary metabolites from panchagavya samples drawn at different intervals were out sourced for carrying out GCMS analysis. A total of 105 compounds were detected and characterized. However, metabolites that showed more than 60 per cent purity with a minimum retention time (RT) of 7 minutes were considered in this study. A total of 36 such secondary metabolites in panchagavya samples drawn at different intervals detected are compiled in Table 12.

Panchagavya samples collected at various stages were subjected to GCMS analysis. The GCMS analysis of the volatile compounds present in the panchagavya extracts were determined by mass spectra and retention time analysis through GCMS. Compounds identified through GCMS analysis revealed that metabolites mainly comprised of fatty acids, esters, acids, alcohols and hydrocarbons. The GCMS analysis reported 36 volatile compounds, found in panchagavya sampled at 4 different intervals. Some compounds were found at only one stage and some were found across all the stages of panchagavya.

Compounds such as 1, 2-Diaminoethane, 1, 2-bis 2-hydroxyphenyl, 1-Nonadecene, Tridecanoic acid methyl ester and Heptadecanoic acid methyl ester were the five compounds present only in samples drawn on 7^{th} day. Octanoic acid was present in sample drawn on 14^{th} day. Octanoic acid methyl ester, Hexanoic acid butyl ester, Benzoic acid, 1,5-Hexadiyne, Ethyl Oleate, Hexadecanoic acid butyl ester and Trans-13-octadecenoic acid these seven compounds were detected in samples dawn on 21^{st} day. Heneicosanoic acid methyl ester, Butyl myristate and 11-Dodecenoic acid 10-hydroxy-methyl were the three compounds present only in sample drawn on 30^{th} day. Hexadecanoic acid methyl ester and Pentadecanoic acid methyl ester were the two compounds present in all the stages of panchagavya (Table 12). All these compounds with their known biological activity have been listed in Table 13.

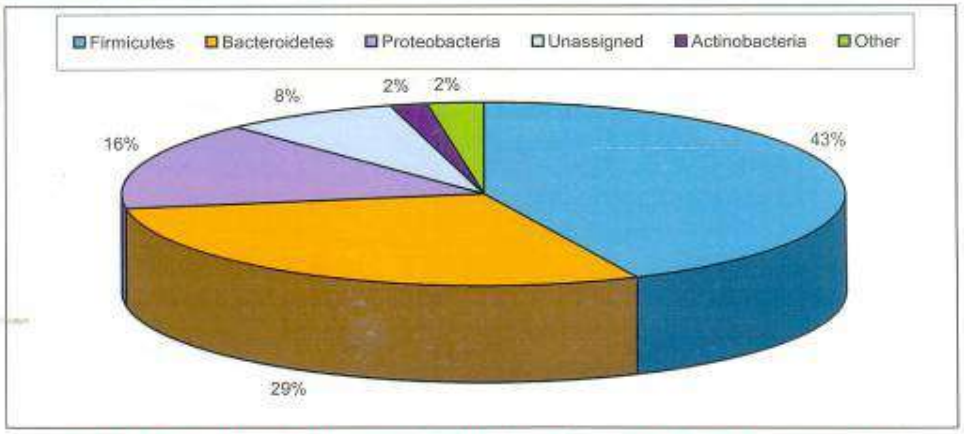


Fig. 8. Phylum level taxonomic distribution of bacteria in the metagenome of panchagavya sample drawn on 14th day

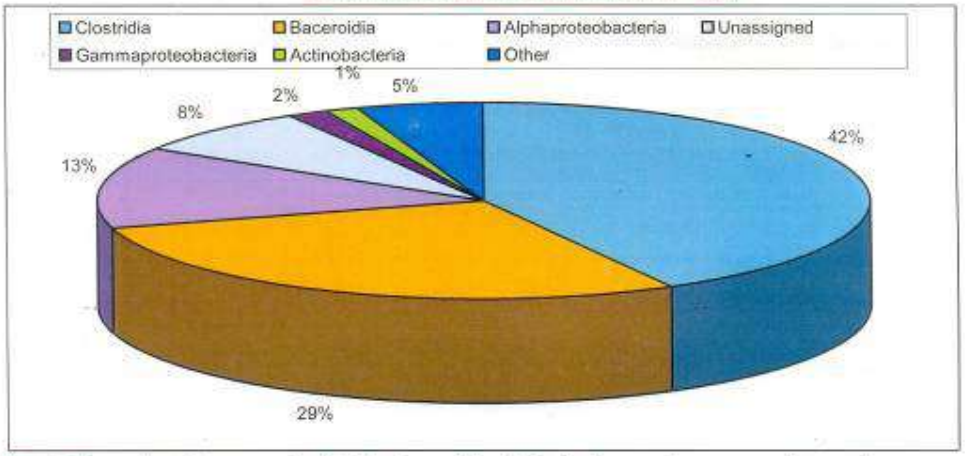


Fig. 9. Class level taxonomic distribution of bacteria in the metagenome of panchagavya sample drawn at 14th day

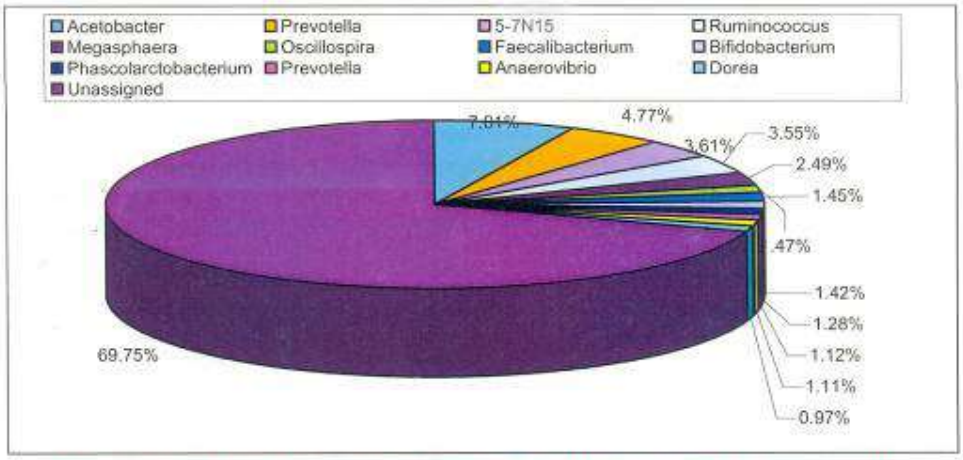


Fig. 10. Genus level taxonomic distribution of bacteria in the metagenome of panchagavya sample drawn at 14th day

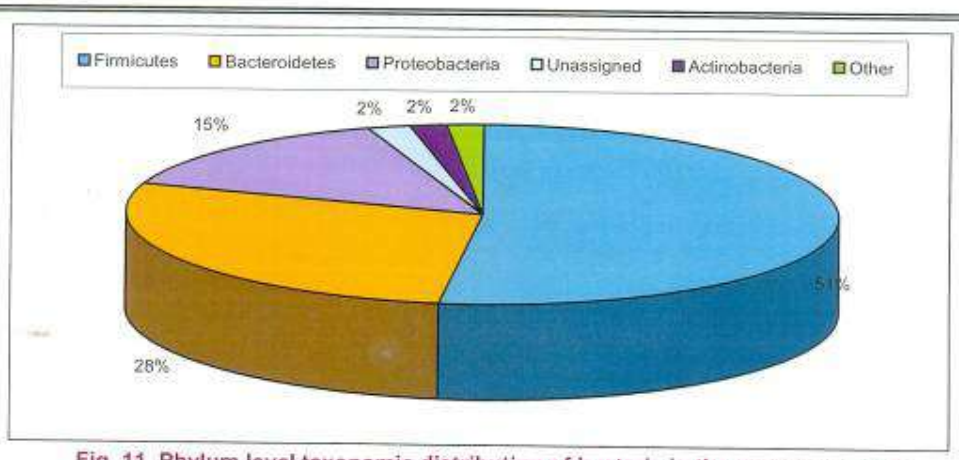


Fig. 11. Phylum level taxonomic distribution of bacteria in the metagenome of panchagavya sample drawn at 21st day

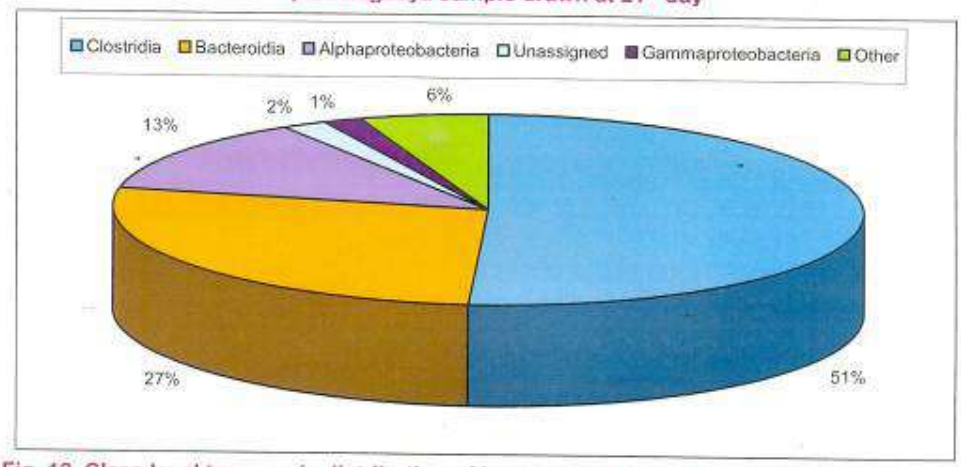


Fig. 12. Class level taxonomic distribution of bacteria in the metagenome of panchagavya sample drawn at 21st day

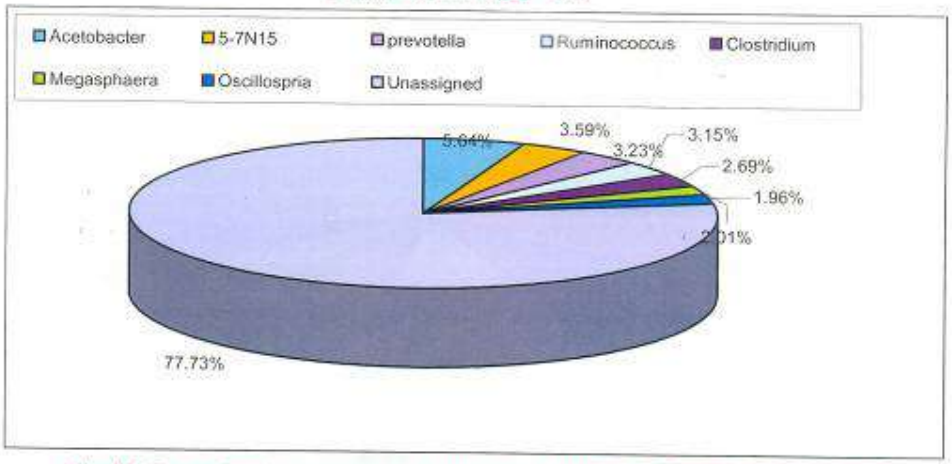


Fig. 13. Genus level taxonomic distribution of bacteria in the metagenome of panchagavya sample drawn at 21st day

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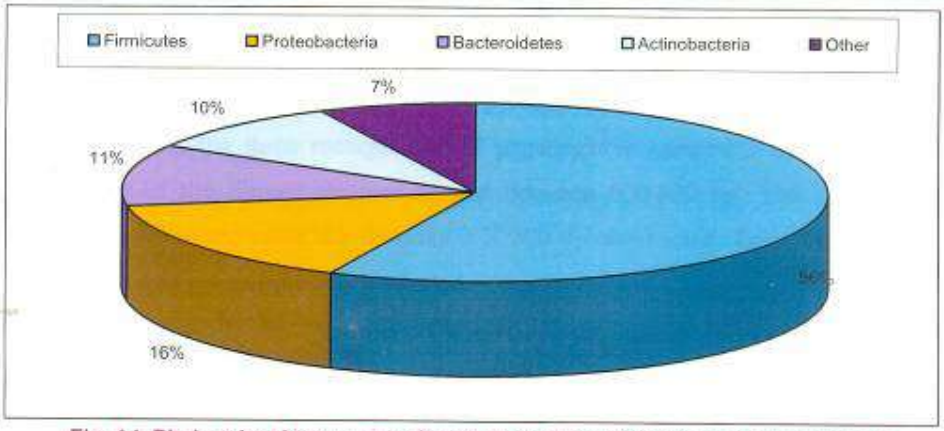

Fig. 14. Phylum level taxonomic distribution of bacteria in the metagenome of panchagavya sample drawn at 30th day

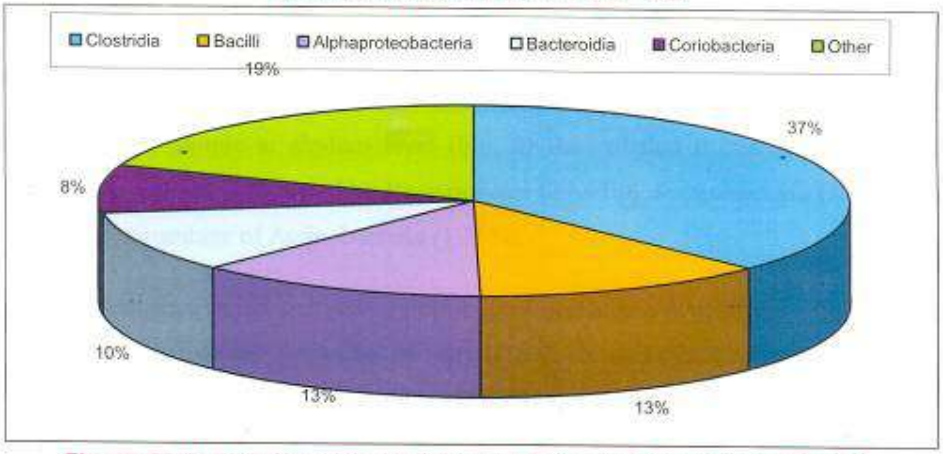

Fig. 15. Class level taxonomic distribution of bacteria in the metagenome of panchagavya sample drawn at 30th day

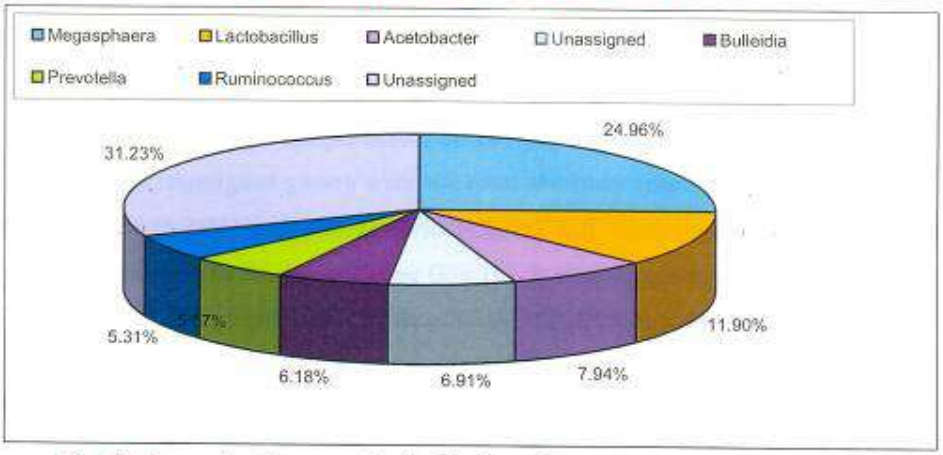

Fig. 16. Genus level taxonomic distribution of bacteria in the metagenome of panchagavya sample drawn at 30th day

4.7 Library preparation and pyrosequencing

4.7.1 Qualitative and quantitative analysis of Genomic DNA

High quality genomic DNA was observed on 0.8 per cent agarose gel and the average DNA yield for 14th, 21st and 30th day panchagavya samples were 210, 179 and 194 ng/µl respectively at 260/280 nanodrop reading.

4.7.2 Construction of metagenomic library

Metagenomic libraries of V3-V4 partial region of 16S rRNA gene segment amplified from the three metagenome of panchagavya samples were prepared. The mean size of the library obtained ranged between 600-630 bp. The libraries were sequenced in MiSeq using the Illumina 2 X 300 bp sequencing. From this about 150-250 Mb of data per sample was generated.

4.7.3 Bioinformatics analysis

QIIME bioinformatics software was used for analyzing the data generated from 16S rDNA metagenome sequencing. The results with the different barchart, piecharts and figures at different levels are shown below.

The results of squence based analysis and taxonomic distribution of 14th day panchagavya sample at phylum level (Fig. 8) showed that it contained majority of Firmicutes (43.09%) followed by Bacteriodetes (29.40%), Proteobacteria (15.60%) and very less percentage of Actinobacteria (1.75%).

Sequence based analysis and class level taxonomic distribution of metagenome of panchagavya sample drawn on 14th day (Fig 9) showed that it contained majority of microbes belonging to class Clostridia (42.03%) and Bacteroidia (28.65%) followed by Alphaproteobacteria (13.23%) and unassigned class of bacteria (7.71%). Also, a very low percentage of Gammaproteobateria (1.80%) and Actinobacteria (1.44%) were recorded.

Sequence based analysis of metagenomic library for taxonomic distribution of bacteria in panchagavya sample drawn on 14th day at genus level revealed that bacteria belonging to unassigned genera were the most abundant (nearly 76.72%), followed by *Acetobacter* (5.25%) and *Anaerovibrio* (1.07%) and the lowest per cent (1.06%) of bacteria belonged to the genus *Dorea* (Fig.10). It was interesting to know that bacteria belonging to Genera *Oscillospira, Ruminococcus, Bifidobacterium* were also found to be occurring in very low percentage.

Sequence based analysis and phylum taxonomic distribution in metagenome of panchagavya sample drawn on 21st day (Fig.11) indicated that

it contained majority of Firmicutes (51.85%) and Bacteroidetes (28.03%) followed by Proteobacteria (14.77%), some amount of Actinobacteria (1.63%) and unassigned bacteria (1.92%).

Class level taxonomic distribution of bacteria in the metagenome of panchagavya sample drawn on 21st day showed that the sample contained majority of bacteria belonging to class clostridia (50.70) and Bacteroidia (27.28%) followed by Alphaproteobacteria (12.79%) and Gammaproteobacteria (1.47%). While, bacteria belonging to unassigned class was about 1.92 per cent.

The genera level taxonomic distributions in the metagenome of panchagavya sample drawn on 21st day revealed that most of the bacteria were from unassigned genus (77.73%). While 5.64 per cent belonged to *Acetobacter* followed by *5-7N15* (3.59%), *Prevotella* (3.23%). Some percentage of *Ruminococcus* (3.15%), *Clostridium* (2.69%) and *Megasphaera* (2.01%) were also reported (Fig.13).

Taxanomic distribution of bacteria in 30th day drawn panchagavya sample at phylum level revealed that the major phylum was Firmicutes (56.57%) followed by Proteobacteria (15.77%), Bacteroidetes (10.88%), Actinobacteria (9.51%) and other bacteria (7.27%) (Fig. 14).

Taxanomic distribution of bacteria at class level present in the metagenome of panchagavya sample drawn on 30th day at class level showed that majority of bacteria belonged to class Clostridia (37.06%) followed by Bacilli (12.86%), Alphaproteobacteria (12.62%) Bacteroidia (10.18%) and Coriobacteria (8.13%). However, 19.50 per cent of bacteria were observed to be grouped as belonging to other class (Fig.15).

Taxanomic distribution of bacteria at genus level in the metagenome of panchagavya sample drawn on the 30th day showed that most of the bacteria belonged to unassigned genus (31.23%). Followed by genus *Megasphaera* (24.96%), *Lactobacillus* (11.90%), *Acetobacter* (7.94%). Also, genus *Bulleida* (6.18%), *Prevotella* (5.57%) were observed at low percentage (Fig.16).

Comparative analysis of taxonomic abundance of bacteria in three stages of panchagavya at phylum level was carried out (Table 14a). The results showed that the highest number of bacteria were under phylum Firmicutes. This was followed by phyla; Actinobacteria and Proteobacteria in the 30th day panchagavya sample. Only Bacteroidetes were observed at the highest level in 14th day panchagavya sample.

Comparative analysis between the metagenome at three stages of panchagavya samples at class (Table 14 b) and genus (14c) level were carried out to identify dominant taxa of bacteria. The results revealed that class Clostridia, Bacterroidia, Alphaproteobacteria, Gammaproteobacteria and bacteria belonging to unassigned class were observed as the highest occurring class in the metagenome of panchagavya sample drawn on 14th day as compare to that of 21st and 30th day panchagavya samples.

Genus level comparative analysis for bacteria occurring at different stages of panchagavya making was also carried out (Table 14c) using QIIME bioinformatics software. The result showed that genus *Megasphaera, Lactobacillus, Bulleidia* and *Rumminococcus* were represented with higher percentage in panchagavya sample drawn on 30th day as compare to 14th and 21st day. While the genera *5-7N15* and *Acetobacter* were observed in high percentage on 15th day sample of panchagavya.

Rarefaction curve helps in calculating species richness for a given number of individual samples, based on the construction. Rarefaction curve for panchagavya sample drawn on 14th, 21st and 30th day (Fig. 17) provided the information of the sequence coverage obtained with the amplicon sequencing technique. The plateau of the curve is an indication of the species count (number of OTUs) on Y axis in relation to the number of reads generated (numerical threshold of sequences) on X axis of the corresponding (OTUs).

The maximum species richness was observed in the sample drawn on 14th day (855 species with a maximum sequence read of 252000, followed by the sample drawn on 30th day with approximately 637 species with sequence read of

Table 14a: Comparative analysis between the samples at phylum level

Phylum	14th day sample	21st day sample	30th day sample
Firmicutes	43.10 %	51.60 %	56.60 %
Bacteroidetes	29.40 %	27.50 %	10.80 %
Proteobacteria	15.60 %	14.70 %	15.70 %
Unassigned	7.70 %	2.90 %	7.00 %
Actinobacteria	1.80 %	1.60 %	9.50 %
Other	2.40 %	1.70 %	0.40 %

Table 14b: Comparative analysis between the samples at class level

Class	14th day sample	21st day sample	30th day sample
Clostridia	42 %	50.40 %	37.10 %
Bacteroidia	28.60 %	26.70 %	10.20 %
Alphaproteobacteria	13.20 %	12.70%	12.60 %
Unassigned	7.70 %	2.90 %	7.00 %
Gammaproteobacteria	1.80 %	1.50 %	1.00 %
Actinabacteria	1.40 %	1.30 %	1.40 %
Other	5.00 %	4.50 %	30.60 %

Table 14c: Comparative analysis between the samples at Genus level

Genus	14th day	21st day	30th day
Megasphaera	1.60 %	2.00 %	24.90 %
Lactobacillus	0.30 %	0.40 %	11.70 %
Bulleidia	0.10 %	0.10 %	6.30 %
Ruminococcus	2.70 %	3.10 %	5.30 %
Unassigned	7.70 %	2.90 %	7.00 %
Acetobacter	5.20 %	3.00 %	1.90 %
Prevotella	4.00 %	3.20 %	5.60 %
5-7N15	3.90 %	3.60 %	1.70 %
Unassigned	74.50 %	81.40 %	35.60 %

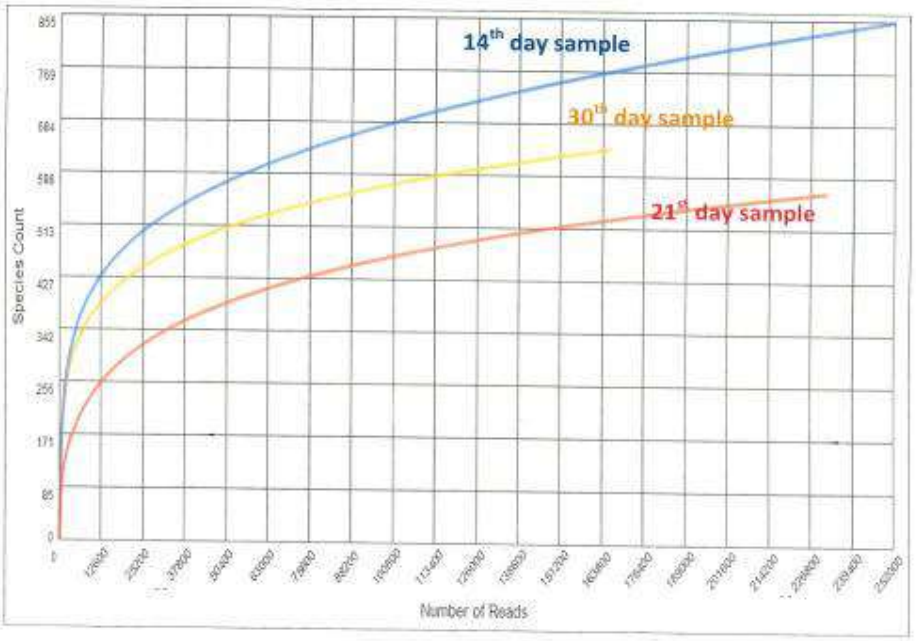

Fig. 17. Rarefaction curve drawn from the sequence data of panchagavya metagenome samples drawn on 14th, 21st and 30th day of preparation

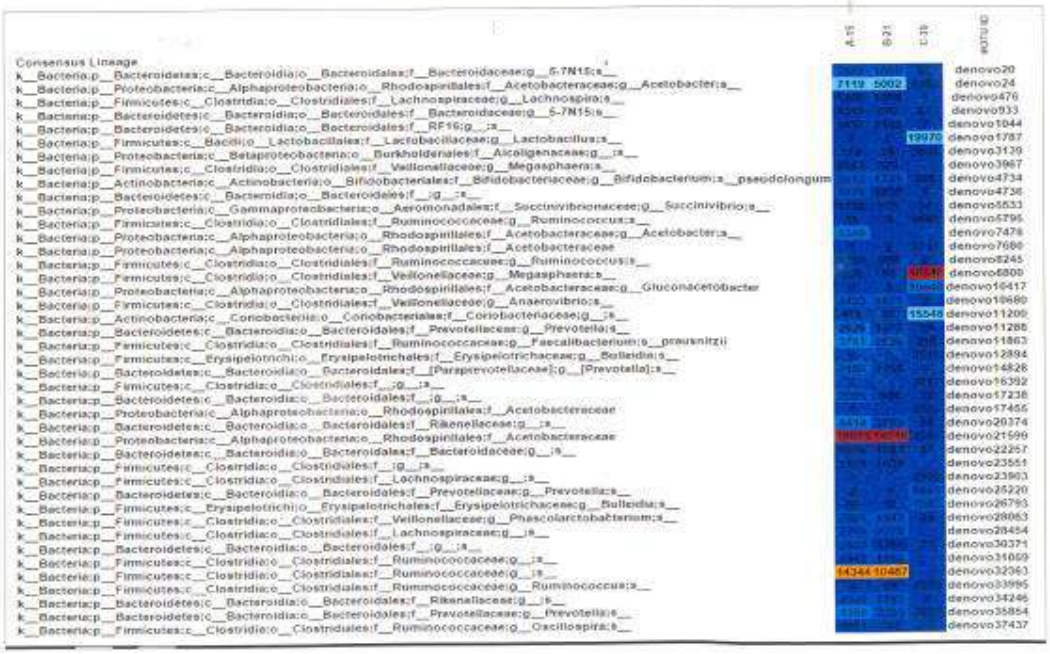

Fig. 18. Heat map showing assigned taxonomy for each OUT present from the metagenome of panchagavya samples drawn on 14th, 21st and 30th day

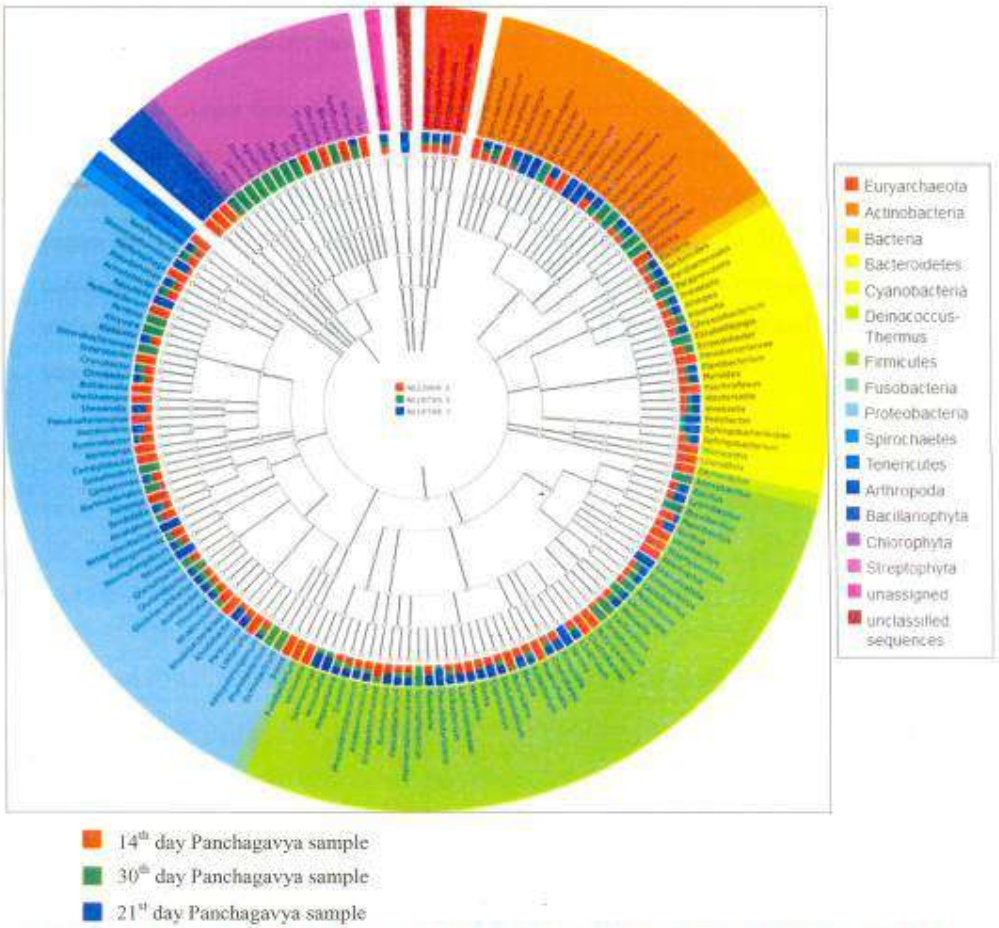

Fig. 19. Lowest Common Ancestor (LCA) plot at phylum and genus level of bacterial community in the metagenomes of panchagavya samples drawn on 14th, 21st and 30th day of preparation

163800 while the sample drawn on 21st day had the least species richness (approximately 576 species with a sequence read between 228800 and 239400).

The OTU heatmap displays raw OTU counts per sample. The counts are coloured based on the contribution of each OTU to the total OTUs count present in that sample (blue: contributes low percentage of OTUs to sample; red: contributes high percentage of OTUs). The table based on taxonomy assignment was filtered to a OTU table restricting to 2000 numbers of counts per OUT (Fig. 18). *Acetobacteriacea, Megasphaera and Ruminococcus* are the three genera known to be in very high percent in all the metagenomes screened.

It is evident from the plot that bacteria representing as many as 17 phyla could be detected in the metagenomes of panchagavya samples drawn at different intervals during its preparation. The taxons with red colour are unique to panchagavya sample drawn on 14th day. Similarly, green coloured taxa are unique to 30th day and taxa coloured blue are unique to 21st day drawn panchagavya sample. Correspondingly, taxa with more than one colour are known to occur at more than one stage and taxa with all three colours are common at all stages of panchagavya preparation.

5. DISCUSSION

Modern day agriculture employing costly external inputs is confronted with loss of soil health and environmental quality. Farming systems where in nutrient sources and other organic inputs are met from balanced use of natural resources appear to provide sustainable crop production avenues over years. These are also presently attracting the attention of farmers. A number of indigenous nutrient sources such as organic manures, enriched manures, vermicompost, green manures and also liquid organic manures are being used as natural sources of nutrients. Liquid organic manures such as *Beejamruth, Jeevamruth, Amruthpani* and *Panchagavya, etc.,* are very popular among organic farmers. Among these panchagavya derived from five different products of cow is the most popular and widely used liquid organic source of nutrient. The beneficial effect of panchagavya is attributed to the known beneficial effects of cow products used in its making and the microbial process involved in its preparation. Existing scientific knowledge clearly suggest that it contains nutrients and growth promoting substances that accrue during various stages of preparation and hence when applied to crops it is known to improve growth and yield of crops. An attempt was made to understand the microbial process involved in its preparation using culture independent techniques. The metagenomes of panchagavya sampled at different stages of making and also from the phylloplane sprayed with it were probed for understanding the bacterial diversity. Specific primers were used to amplify bacterial ribosomal genes and analyzed using DGGE. A number of diversity indices such as Shannon diversity index, Range weighted richness, Sorenson similarity index, Pielous evenness index were computed from the data. Further, moving window analysis and Pareto Lorenz curve were developed and metagenome library were sequenced to understand microbial diversity involved with panchagavya preparation and its application to crops. The results obtained and the interpretations of the results in the light of set objectives of this study are discussed in this chapter.

The conventional techniques involving culture dependent methods such as the cultivation of microbes, phenotypic characterization and PCR-based approaches are often met with a limitation of accessing only one to five per cent of bacterial diversity from environmental samples. The present study aimed at accessing the

metagenome of panchagavya to understand bacterial diversity using culture independent techniques, construct libraries of 16S rDNA genes amplified from metagenome and sequencing them was attempted for the first time. Metagenomic analysis of cow's products such as dung, milk and curd were also carried out in this study. Previously, isolation of metagenomes from cow dung (Girija *et al*., 2013), cow milk and curd (Giannino *et al*., 2009) and their analysis was done successfully.

5.1 Isolation of metagenomic DNA from Panchagavya, individual ingredients and phylloplane

The metagenomic DNA from panchagavya at different stages of preparation and from cow dung (Kim *et al.,* 2010), cow milk (Lopez *et al.,* 2004) and curd (Massana, *et al.,* 1997) and phylloplane (Delmotte *et al*., 2009) was isolated with slight modification to protocols earlier followed for other samples. The modifications to the protocols were tried in different concentrations and standardized. The additional steps included in addition to DNA extraction buffer of 100 mM $CaCl_2$, 100 mM EDTA and 1.5 ml of 20% SDS to the sample. There in the present study was found very effective to obtain metagenome with high purity. This method yielded metagenomic DNA in the range of 1150- 1635 ng/µl from panchagavya, 1550- 1820 ng/µl from dung, 550- 920 ng/µl from curd 350- 580 ng/µl from milk and 800 - 1214 ng /µl from phylloplane with a purity ratio of 1.78-1.87.

Same modification with the protocols for extraction of metagenomic DNA from cow dung, cow milk, curd and phylloplane yielded a high quality metagenomic DNA. It had been reported by Zhou *et al.* (1996) that $CaCl_2$ and EDTA concentrations when standardized and used in the protocols for isolation of metagenomic DNA resulted in higher yield of DNA with greater purity. The metagenomic DNA isolated from phylloplane of soybean plants using the modified protocol also yielded high quality as revealed on 0.8% agarose gel. This type of high quality metagenomic DNA is a prerequisite for downstream process as suggested by Clegg *et al.* (1997). Since, the DNA obtained was highly pure it was diluted at 1:5 ratio before using it for PCR amplification. Similarly, diluted metagenomic DNA had been used in PCR reaction by Borneman *et al*. (1996). The aim of present study was to understand bacterial diversity in the metagenomes of panchagavya, phylloplane, cow dung, cow milk and curd.

5.2 PCR amplification of isolated metagenomic DNA

In prokaryotes, the 16S ribosomal RNA (rRNA) genes are the most conserved and are known to occur at least in one copy per genome (Acinas *et al*., 2004). The universality of the genes makes them an ideal target for phylogenetic studies and taxonomic classification. Universal primers targeting 16S rRNA genes were employed as suggested by Gray *et al* (1999). Primers targeting V1 to V9 regions were used previously for species identification (Lane *et al*., 1985). Earlier experiments were successfully used primers targeting V3-V5 and V6 regions of 16S rDNA and analyzed them using DGGE and pyrosequencing (Armougom and Raoult, 2009). These three variant regions in 16S rDNA have provided sufficient phylogenetic information about bacteria in the samples (Huse *et al*., 2008 and Liu *et al*., 2008). Therefore, in the present study the first set of primers used (PRBA338-PRUN518) targeted the V3 region of 16S rDNA as suggested by Nakatsu *et al*. (2000). The second set of primers (A905-A1059) specifically targeted the V6 region of archaeal 16 S rRNA and the third set of primers (E783-E926) targeted V5 region of eubacterial 16S rRNA (Huws *et al*., 2007) using PCR for amplification. The primer set; PRBA338-PRUN518 was found useful to amplify around 180 bp in V3 region, primer set; E783-E926 provided amplicons of about 143 bp in V5 region and the primer set A905-A1059 generated about 154 bp sized amplicons of V6 region. The choice of the primers and the reaction conditions for PCR amplification of 16S rDNA variable region was found to be 96% efficient in providing the expected targeted region and also yielded good amplicons from genomic DNA as revealed on 0.8% agarose gel (Plate 5,6,7). From these it is very clear that a proper combination of the primer and PCR conditions was very crucial to obtain desired amplicons of 16S rDNA from metagenomes in this study.

5.3 DGGE analysis

DGGE is one of the most well established molecular tool in microbiology (Muyzer *et al*., 1999; Boon *et al*., 2002). This can be used to identify bacterial species from natural environment on the basis of variable regions present in their 16S rDNA genes (Dokja *et al*., 2000). The profiles of bands which get separated due

to their GC content are helpful to reveal the bacterial diversity. Each band is considered as OTUs or species which has very high versatility and reproducibility (Prosser *et al*., 2007). The data obtained from DGGE analysis could be used for calculating diversity, similarity, evenness and dynamics of microorganisms present in the metagenomic DNA of sample. These data provide an insight into community structure, population and dynamics which remain obscure by other traditional methods (Prosser *et al*., 2007). In this study satisfactory DGGE profiles revealing the bacterial diversity in the metagenome of panchagavya samples, phylloplane and individual ingredients were obtained (Plate 8, 9, 14 and 15). The pattern of DGGE after staining were analyzed using SynGene tool a software that clearly distinguished and scored bands based on their position and intensity in the gel. The data was used to calculate diversity indices.

5.3.1 Shannon diversity

A Shannon diversity (H) index is a mathematical measure of species diversity in a given community based on the species richness (the number of species present) and species abundance (the number of individuals per species) (Shannon, 1948). This index provides important information about rarity and commonness of species in a community. When both diversities and richness increase Shannon diversity index value also increases. In the present study the highest Shannon diversity index (H) was observed in the panchagavya sample drawn on 14^{th} day (2.96) followed by the sample drawn on 21^{st} day (2.82) indicating that panchagavya samples of 14^{th} and 21^{st} days were having more species richness and species abundance while the lowest value of 2.35 was observed in metagenome of panchagavya sample drawn on 30^{th} day (Table 3). Among the three ingredients, cow dung showed the highest Shannon diversity (3.31) indicating that dung sample had higher species richness and abundance as compared to other ingredients namely milk and curd.

In the preparation of panchagavya in this study ingredients at the starting include 600 gm of dung and 100 gm of ghee along with 400 ml of cow urine and 1000 ml of water and thus higher diversity on 14^{th} day could be attributing mainly due to the bacteria present in the dung. Dung is also an important source of archaeabacteria and their abundance was higher from 3^{rd} day to 7^{th} day, there after gradually declined. It can be inferred from table 3 that among the total bacteria

dominant bacteria in panchagavya till 7th day appeared to be archaeabacteria. However, as the contents were gently agitated twice a day the obligate anaerobic archaeabacterial group such as methanogenic bacteria appeared to have been reduced hence reflected by decline in archaebacterial diversity after 7th day. The aerobic and facultative anaerobic (fermentative) bacteria were likely to have increased from 7th day (2.80) till 14th day (2.96). On 15th day the panchagavya was supplemented with milk, curd, sugarcane juice, ripened banana, cow urine and coconut water. Further, the contents were constantly agitated gently to create aerated condition. Consequently, a shift in the bacterial flora must have caused a drop in Shannon diversity from 14th day (2.96) to 30th day (2.35) for all bacteria. It is also interesting to know that the Shannon index for total bacteria in dung was the highest (3.3) suggesting that both number of species and their abundance was higher in cow dung than milk and curd. The changing values of Shannon diversity index in panchagavya samples drawn at different intervals clearly suggested that both number of species and also number of individual within species changed over time clearly exhibiting bacterial succession in this process.

The Shannon diversity index for bacteria in the phylloplane clearly revealed higher bacterial diversity in phylloplane sprayed with panchagavya as compared to unsprayed phylloplane. Spraying was also helpful to increase the diversity of archaeabacteria in phylloplane.

5.3.2 Range weighted richness

The species richness in the metagenomic DNA was calculated based on the number of bands (OTUs) present per sample within 30- 70 per cent denaturing gradient. The critically analyzed bands were used to calculate species richness and expressed as range weighted richness (Rr). The Rr value of more than 30 implies a typical and very habitable environment with broad carrying capacity, high microbial diversity and high range weighted richness. Panchagavya was prepared using cow dung and ghee at the beginning until 15th day and milk, curd, coconut water, ripened banana, sugarcane juice were added after 15th day. These ingredients are either rich source of nutrient and or have inherently abundant bacterial flora which could have contributed to higher weighted richness in panchagavya samples at all the stages. Dung as a rich source of nutrient and bacteria has been reported by Girija *et al*. (2013). Maheshwari *et al*. (2007) opined that diverse microflora in panchagavya

might come from the ingredients such as dung, milk and curd. In this study the bacterial diversity in the metagenomes of dung, milk and curd was assessed. It was found that each one of them had distinct as well as common microflora. Consequently, panchagavya at different stages was known to share about 31 per cent similarity of total bacteria. While the similarity in bacterial populations between panchagavya and dung was 35 per cent, with milk 21 per cent and with curds 9 per cent. The bacterial similarity shared between dung and milk was 20 per cent, dung and curd 12 per cent and milk and curd was 13 per cent. From all these it could be inferred that the bacterial diversity in panchagavya accrued because of the diverse groups of bacteria present in each of the individual ingredients used in its making.

The three ingredients of panchagavya also had higher value of range weighted richness which implied that they carried broad groups of bacterial flora showing species richness within each of it. The highest bacterial richness was seen in the cow dung (556.87) with an archaebacterial richness of 421. This was much higher than the eubacterial richness (66), and together these two resulted in a very high total bacterial richness in the dung. While in cow milk and curd it was interesting to know very high archaeabacterial richness which needs to be explained with techniques having greater sensitivity.

Similarly, the value of range weighted richness for total bacteria and archaea was higher in phylloplane irrespective of spraying at all stages. However, higher richness value was documented in phylloplane sprayed at all the stages as compared to those without spraying. This clearly suggested that panchagavya which also a rich source of bacteria when sprayed to phylloplane selectively increased richness of bacterial species in the phylloplane.

5.3.3 Sorenson's pair-wise similarity index

It is a measure of similarity in the bacterial composition between samples. A similarity index of 100 per cent indicated that DGGE profiles were identical while completely different profiles resulted in a value of 0 per cent. Sorenson's similarity index of panchagavya sample with PRBA338-PRUN518 primer pair showed that the sample drawn on 14^{th} and 21^{st} day shared the highest similarity of 65 per cent indicating that 65 per cent of the bacterial species in both the samples were common. This was followed by sample drawn on 3^{rd} and 7^{th} days sharing 55 per

cent similarity. However, samples drawn on 14th and 30th days shared only 14 per cent of species similarity (Table 7). This is likely because fresh ingredients containing native bacteria and or nutrients were added on 15th day which could have caused changes in microbial community structure and numbers in the panchagavya.

Further, as evident from Shannon diversity index the archaeabacterial richness showed a reduction from 7th day till 30th day. However, the total bacterial diversity was found to increase from 7th day to 14th day there after slowly declining. Similar trend was also observed with archaeabacterial and eubacterial population in 14th and 21st day samples of panchagavya. It was interesting to note that 44 per cent similarity existed in total archaebacteria at all stages of panchagavya preparation. While the similarity of archaebacteria amongst panchagavya cow milk, cow dung and curd ranged between 32-34 per cent. The notable similarity of archaeabacteria at various stages of panchagavya could be attributed to addition of cow milk and curd which also contained higher diversity of archaebacteria as evident from Shannon diversity index value (Table 3). Small fluctuations and drop in archaebacterial populations could be caused due to changes in redox potential in the system due to agitation which could have affected the well known obligate anaerobic methanogenic bacteria in microbiomes within the system.

5.3.4 Pielou's evenness index

Pielou's evenness value for all the stages of panchagavya along with their individual ingredients recorded values of more than 0.75 per cent for both bacterial and archaeal specific primer pairs PRBA338-PRUN518 and A905-A1059 respectively (Table 10). Results showed that species were more evenly distributed at all the stages of panchagavya including their individual ingredients. Pielou's evenness index value of one and closer to it indicated highly even distribution of species in sample and index value of zero and closer values indicate highly uneven distribution of species in a sample (Pielou, 1966).

Panchagavya sprayed samples showed higher even species distribution as compared to unsprayed control samples. Spraying panchagavya at 15th and 30th days resulted in higher even distribution of species as compared to control and panchagavya sprayed at only one time (Table 11). This is possibly because spraying of panchagavya second time (15th day followed 30th day) could have added similar

species of microbes in panchagavya and thus contributing to greater evenness of species.

5.3.5 Functional organization (FO)

Pareto Lorenz curve drawn for samples of panchagavya from different stages, individual ingredients (cow milk, curd and dung) and phylloplane showed that the curve values for all the samples were more than 80 per cent on Y axis. At 20 per cent intercept on X- axis this implies that a small amount of the species in each sample is dominant and all the others were present in low numbers (Lorenz, 1905). It is also likely that a specialized microbial community could exist in each sample (Fernandez *et al*., 1999). As observed by Massimo *et al*. (2008) in their study this community could be highly functionally organized but fragile to external changes because disruption might require longer recovery times for microbes to maintain their favourable environment.

5.3.5 Microbial dynamics

Dynamics of a microbial community in a sample is a measure of the average rates of change in parameter and degree of change between consecutive DGGE profiles of the same community over a fixed time interval (Nauhaus *et al*., 2007). Based on moving window analysis, the rate of change (D*t*) in parameter had been calculated during all the consecutive stages of panchagavya at defined time interval using PRBA338-PRUN518, A905-A1059 and E783-E926 primer pairs. It was observed that the rate of change in parameters with the three primers used were 17.8, 8.51 and 10.90 per cent respectively for PRBA338-PRUN518, A905-A1059 and E783-E926 primer pairs. Overall 13 per cent change was observed in all the samples for all the three primers used. This situation was assumed to represent a medium level of dynamics present between different stages of panchagavya. Further, it could imply that in each sample a new species entered into pre existing bacterial community but did not interfere with the functionality of the pre-existing population as observed by Miura *et al.* (2007) in their studies.

Average Dt value of 4.96 per cent were observed among unsprayed phylloplane samples at different intervals which could be attributed as low bacterial dynamics. While in the sprayed phylloplane sample Dt value of 11.08 per cent was observed reflecting average rate of bacterial change in these samples. These kinds of environments are classified under environments eco-friendly to come and colonies by other type of species (Massimo *et al.,* 2008).

5.4 Gas Chromatography Mass Spectroscopy (GCMS) analysis of secondary metabolites present in panchagavya sample The report of the outsourced GCMS analysis of panchagavya samples drawn at different intervals has identified 36 volatile compounds, out of which 16 compounds were known to have one or the other biological activity (Table 13). Compounds such as 1,2-Diaminoethane, 1,2-bis 2-hydroxyphenyl, 1-Nonadecene, Tridecanoic acid methyl ester and Heptadecanoic acid methyl ester were present only in samples drawn on 7th day.

Octanoic acid was present in sample drawn on 14th day. Octanoic acid methyl ester, Hexanoic acid butyl ester, Benzoic acid, 1,5-Hexadiyne, Ethyl Oleate, Hexadecanoic acid butyl ester and Trans-13-octadecenoic acid were the seven compounds detected in samples drawn on 21st day. Heneicosanoic acid methyl ester, Butyl myristate and 11-Dodecenoic acid 10-hydroxy-methyl ester were the three compounds present only in the sample drawn on 30th day. Hexadecanoic acid methyl ester and Pentadecanoic acid methyl ester was the two compounds present in panchagavya samples of all the stages (Table 12).

The GCMS analysis of panchagavya samples drawn at various stages revealed presence of Pentadecanoic acid methyl ester, Ethyl Oleate and Hexanoic acid. These compounds have been reported for antibacterial and antifungal activities (Sermakkani and Thangapandian, 2012). Heptadecanoic acid methyl ester, Pentanoic acid, Trans-13-Octadecenoic acid were reported for their antioxidant biological activity (Agoramoorthy *et al*., 2007). Hexadecanoic acid butyl esters and Trans-13-Octadecenoic acid had been reported for antitumor activity (Khalaf *et al*., 2014). n-Hexadecanoic acid methyl ester has been reported for nematicidal activity (Omotoso *et al*., 2014). Some of the compounds were Hexadecanoic acid methyl ester, 9-Octadecenoic acid (Z) methyl ester, Octanoic acid methyl ester, Oleic acid and n-Hexadecanoic acid methyl ester reported for more than one biological activity. Cetene, 1-Nonadecene, Methyl myristoleate, Methyl 8-methyl-nonanoate, Benzene

acetic acid methyl ester and Methyl stearate were compounds detected in the sample but their biological activity not known (Table 13). It was interesting to know that in 7 day old sample of panchagavya there were four compounds with biological activity while in 14 days old sample this number was six. The number of biologically active compounds detected in panchagavya samples of 21 and 30 days were 13 and 5 respectively. Only two compounds namely; Hexadecanoic acid butyl ester with biological activity (anti-oxidant, nematicide, five alpha reductase inhibitor and pesticide) and Pentadecanoic acid, methyl ester a known anti-oxidant were present in panchagavya samples drawn at different intervals.

5.5 Bioinformatics analysis data generated from pyrosequencing

Taxonomic positions of bacteria in all panchagavya samples drawn at different intervals was dominated by phyla Firmicutes followed by Bacteroidetes and Proteobacteria. Further the dominant classes were Clostridia, Bacteroidia, Alphaproteobacteria and Actinobacteria. The predominat genera assigned were *Megasphaera, Lactobacillus, eulleidia, Ruminococcus* and *Acetobacter.* Within phylum Firmicutes the bacteria belonging to *Clostridium*, *Rumminococcus* and *Bacillus* genera were identified. *Clostridium* is a known broad genus ubiquitous in the gastrointestinal track. *Clostridia* can have both negative and positive influence in an environment. They are beneficial to improve digestion of organic materials such as cellulose, hemicelluloses, xylose, chitin and lignocelluloses. They also act as beneficial probiotics and nitrogen fixer (Mertens *et al*., 2005).

Panchagavya contained predominantly, Proteobacteria followed by Actinobacteria. Similar types of results were observed in organic soil wherein the predominant bacterial phylum were Proteobacteria followed by Actinobacteria (Chan *et al*., 2008). They also reported that *Alpha-* and *Gamma-*P*roteobacteria* were major dominant microflora found in forest soil which was considered as an environment with very high bacterial diversity with species richness. It is difficult, to make a comparisons with other studies in case of phylloplane of plants sprayed with panchagavya, especially because of the differences in the environmental conditions, microbial characterization and vegetation. But in general panchagavya spray to phylloplane caused an enormous increase in the level morphological, physiological and metabolic diversity (Kersters *et al*., 2006). They have reported that when panchagavya was applied to plants through spray or through irrigation the

Proteobacteria present in panchagavya would play significant role in global C, N and S cycling (Kersters *et al*., 2006).

In the 30 days old panchagavya sample, *Actinobacteria* and *Acidobacteria* were seen. Actinobacteria constitute one of the largest phyla among Bacteria and represents Gram-positive bacteria with a high G+C content (Williams *et al*., 1983). Actinobacteria are known to be widely distributed in terrestrial ecosystems, with variable physiological and metabolic properties, which enable them to decompose and mineralize naturally occurring compounds such as cellulose and chitin (Lacey *et al*., 1996). Therefore, they play a crucial role in organic matter turnover. Actinobacteria present in panchagavya may play a major role in improving the surrounding environment by degrading high-molecular-weight organic compounds into readily available organic carbon and energy sources for other microbes to use as carbon sources for growth and survival. Acidobacteria are also reported to play a similar role in carbon turnover in soil (Hugenholtz *et al*., 1998; Barns *et al*., 1999; Eichorst *et al*., 2011).

Species belonging to *Acidobacteria* are known to withstand extreme environments and play important role in nutrient recycling (Ward *et al*, 2009). Soil pH may be one of the important factors for large proportion of *Acidobacteria* observed in inorganic soil. Similarly, panchagavya also has acidic pH and hence could be a very good environment for the occurrence of *Acidobacteria*. This is likely to be the cause for occurrence of this genus in very high number in panchagavya sample drawn on the 30^{th} day.

Taxonomic abundance of comparative analysis between three stages of panchagavya at phylum level showed that the highest number of Firmicutes, Actinobacteria and Proteobacteria were observed in 30^{th} day panchagavya sample. Only Bacteroidetes were observed as the highest group in the 14^{th} day panchagavya sample. Results were indicating that 30^{th} day panchagavya sample environment was congenial for all the types of microbes to colonize and grow.

Results of comparative analysis between three stages of panchagavya sample at class level showed that class *Clostridia, Bacterroidia, Alphaproteobacteria, Gammaproteobacteria* and un-assigned bacteria were predominantly found in the 14^{th} day drawn sample of panchagavya as compare to

21st and 30th day samples. Genus level comparative analysis between three stages of panchagavya helped to know that bacteria belonging to unassigned genus dominated the populations. *Megasphaera, Lactobacillus, Bulleidia* and *Rumminococcus* were observed in higher percentage in 30th day drawn panchagavya sample as compare to 14th and 21st day drawn panchagavya samples. Genus *5-7N15* and *Acetobacter* were observed in higher percentage on 15th day panchagavya sample.

The vast majority of microorganisms from panchagavya have not been cultivated in the laboratory, and almost all of our knowledge of panchagavya microbial life is based on organisms raised in pure culture. The variety of the rest of the uncultured microbial world is staggering and will expand our view of what is possible in biology. The challenge that has frustrated microbiologists for decades is how to access the microorganisms that cannot be cultured in the laboratory. Metagenomic analysis provided an additional set of tools to study uncultured species. This new field offers an approach to study microbial communities as entire units, without cultivating individual members. Metagenomics entailed extraction of DNA from a community so that all of the genomes of microorganism in the community could be pooled. The different hypervariable regions of 16S rRNA gene were amplified using universal bacterial primer pairs. Then the use of next generation sequencing (NGS) covered lot of species from the sample which was useful to study bacteria present in sample panchagavya and its ingredients.

Rarefaction curve for 14th, 21st and 30th day panchagavya sample showed that the curve becomes flatter to the right (Fig.17). This implied that sufficient number of individual from samples had been taken into account and any further intensive sampling most likely would not yield significant additional species. Analysis of the rarefaction curves provided information of the sequence coverage obtained with the amplicon sequencing technique. From this it is evident that the methods followed in this study were sensitive and sufficient data was generated from different analysis based on which meaningful conclusions could be drawn.

Future line of work

- Functional metagenomic approaches could be applied for knowing the genes expressed by functionally organized groups of microorganisms at different stages of panchagavya preparation.
- Metatranscriptomic studies would help in understanding the level of expression of genes present in microflora during each stage of fermentation process.
- Functional metagenomic library can be prepared from panchagavya metagenome and could be used to screen genes with antagonistic activity against insect pests and plant pathogens.
- More sensitive methods could be used to understand the effect of spraying panchagavya to phylloplane of crops and understand the mechanism involved in plant growth promotion due to such sprays.

6. SUMMARY AND CONCLUSIONS

The present research was mainly focused on studying the microbial succession likely to occur at different stages of panchagavya making use of culture independent approaches such as PCR-DGGE and amplicons sequencing. The DNA isolation protocols for all the samples were standardized step by step to develop one working protocol in which addition of $CaCl_2$, EDTA and SDS along with extraction buffer was found useful to obtain high quality metagenomic DNA. Conditions of PCR and the DGGE protocols were also standardized for all the metagenomic samples. Finally, amplicons of 16S rRNA gene for each of the metagenomes were sequenced. The salient findings of this study are summarized below.

1. The protocol for high molecular weight community DNA extraction from panchagavya samples drawn at different intervals, also for cow dung, cow milk and curd were standardized. This helped to obtain a total metagenomic DNA with adequate purity for further analysis.
2. The average yield of metagenomic DNA obtained from panchagavya was in the range of 1150- 1635 ng/µl, while for dung, curd, and milk it ranged between 1550-1820 ng/µl, 550- 920 ng/µl, and 350-580 ng/µl respectively.
3. Metagenomic DNA was also extracted from phylloplane of soybean plants receiving sprays of panchagavya at different intervals along with their respective control and the average concentration of DNA ranged from 800 to 1214 ng /µl.
6. All the DNA samples were diluted to 1:5 ratio and used in PCR as templates. Specific primers for total bacteria, archaebacteria and eubacteria targeting variable region of partial 16S rRNA were used in PCR amplification.
7. Panchagavya samples drawn on 14^{th} day followed by 21^{st} day exhibited the highest bacterial species as measured in terms of Shannon's diversity index and the least in 30 days old panchagavya. Contrarily, archaebacterial diversity was very high up to 7 days and was also the least on 30^{th} day. The diversity in the phylloplane of soybean increased due to panchagavya spray. All panchagavya samples and phylloplane sprayed with panchagavya on 15^{th} and 30^{th} days also showed high weighted richness. Only panchagavya samples drawn on 14^{th} and 21^{st} days shared the highest Sorenson's similarity index (65%). This was closely

followed by samples drawn on 3rd and 7th days with 55% similarity. Pielou's evenness index results indicated that all the samples drawn from panchagavya at different stages along with their individual ingredient showed highly even distribution of bacterial species.

8. Pareto Lorenz curves at 20 per cent interception on X-axis, showed to cover 80 per cent to 95 per cent of bacteria were dominated by a small number of species in each sample.
9. The values depicting the rate of average change in bacterial diversity were 17.8, 8.51 and 10.90 per cent respectively for the three primer sets PRBA338-PRUN518, A-905-1059 and E-783-926 used in this study. This indicated a medium level of dynamics present at the five consecutive stages of panchagavya sampled. While the values depicting these changes between unsprayed (4.96) and sprayed (11.08) phylloplanes were found to have low and medium bacterial dynamics respectively.
10. As many as 36 active volatile compounds ascribed to the groups; fatty acids, esters, acids, alcohols and hydrocarbons were identified in panchagavya samples through GCMS analysis. Three of these compounds were reported to have antibacterial and antifungal activities, three antioxidants, two showing antitumor activity, two with nematicidal activity and five compounds with multiple biological activities were also detected.
11. Comparative analysis of taxonomic abundance between the three stages of panchagavya at phylum level showed that the highest in number of Firmicutes, Actinobacteria and Proteobacteria were observed on 30 days old panchagavya sample. Only Bacteroidetes was observed as the highest phylum in the panchagavya sample drawn on 14h day. The genera level comparative analysis between same samples identified that bacteria belonging to unassigned genus were higher in all the samples as compared to other genera. Genera such as; *Megasphaera, Lactobacillus, Bulleidia* and *Rumminococcus* were observed in higher percentage in 30th day panchagavya sample as compared to panchagavya samples drawn on 14th and 21st days. Genera; *5-7N15* and *Acetobacter* were observed in higher percentage in 15th day panchagavya sample.

12. Rarefaction curve for 14th, 21st and 30th day panchagavya samples showed that the curves tended to become flatter towards the right end side. This implied that sufficient number of species from samples had been taken into account for sequencing and drawing inferences.

Conclusion

This is probably the first research report on the analysis of microbial diversity and succession likely to occur at different stages of panchagavya using a culture independent approach PCR-DGGE and amplicons sequencing. The protocol for high molecular weight community DNA extraction from panchagavya samples drawn at different intervals, also for cow dung, cow milk and curd were standardized. Metagenomic DNA was extracted from phyllosphere of soybean plants receiving sprays of panchagavya at different intervals along with their respective control. All the DNA samples were used in PCR as templates. Specific primers for total bacteria, archaebacteria and eubacteria targeting variable region of partial 16S rRNA were used in PCR amplification. Using DGGE analysis the microbial community composition, diversity, richness, carrying capacity and dynamics present in panchagavya sample from at different stages of panchagavya preparation apart from, individual ingredients and phyllosphere samples were studied. GCMS analysis of panchagavya revealed a total of 36 compounds present in panchagavya samples. Fifteen of these compounds were reported to have one or more biological activity such as antibacterial, antifungal, antioxidants, antitumor and nematicidal activity while the activity of the remaining 21 is yet to be known. Comparative analysis of taxonomic abundance between the three stages of panchagavya at phylum level showed that Firmicutes, Actinobacteria and Proteobacteria were higher in number in 30 days old panchagavya sample. Only, Bacteroidetes was observed as the highest phylum in the panchagavya sample drawn on 14h day. The genera level comparative analysis between panchagavya samples identified that bacteria belonging to unassigned genera were higher in all of them. Genera such as; *Megasphaera, Lactobacillus, Bulleidia* and *Rumminococcus* were observed in higher percentage in 30th day panchagavya sample as compared to panchagavya samples drawn on 14th and 21st days. From this study it is evident that the metagenomes of panchagavya, ingredients and phyllosphere have very specific microbial community with high diversity, high carrying capacity and even distribution of species, showing medium level of dynamics

between the stages of panchagavya. It indicated that small amount of the species in each sample was dominant and all the others were present in low numbers. It is also likely that a specialized microbial community could exist in each sample this community could be functionally highly organized but fragile to external changes because disruption might require longer recovery times for microbes to maintain their favourable environment and function. The findings of this study will open up newer vistas to understand microbial succession and relate it to the microbial metabolites present in

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Appendix I: Preparation of Buffers, Solutions and Enzymes for DNA isolation

1. Preparation of stock solutions

Sl. No.	Name of the component	Concentration in Molar (M)	pH	Quantity for 100 ml (in gm)
1	Tris-HCl	1	8	12.11
2	Sodium chloride	5	-	29.22
3	EDTA	0.5	8	

Note: Maintain pH of by using HCl and NaOH makeup final volume by using millipore water

2. Composition of DNA isolation buffer

Sl. No.	Name of the component	Final concentration in Mm	pH
1	Tris-HCl	100	8
2	Sodium phosphate buffer	100	8
3	Sodium chloride	1500	-
4	EDTA	100	8

Note: The final volume to be made up with Millipore water.

3. Phosphate buffer saline (1X)

Sl. No.	Name of the component	Quantity (g/litre)	Concentration(mM)
1	Sodium chloride (NaCl)	8	137
2	Potassium chloride (KCl)	0.2	2.7
3	Disodium hydrogen phosphate ($Na_2 HPO_4$)	1.44	10
4	Potassium dihydrogen phosphate ($KH_2 PO_4$)	0.27	2

pH 8.0

4. CTAB solution			
Sl. No.	**Name of the component**	**Quantity (100 ml)**	**Concentration**
1	CTAB	2 g	2 %
2	Tris-HCl (1M)	10 ml	100 mm/litre
3	EDTA (0.5 M)	0.2 ml	20 mm/litre
4	Sodium chloride (3 M)	46.67 ml	1.4 M/litre
pH 8.0			

5. Lyzozyme solution (1 ml)		
1	Lyzozyme (50 mg/ml)	10 mg
2	Tris-Cl (10 mM)	1 ml

6. Proteinase K solution (1 ml)		
1	Proteinase K (20 mg/ml)	50 mg
2	Tris –Cl (50 mM)	1000 ml

7. DNAase free RNAase solution (1 ml)		
1	RNAase (10 mg/ml)	10 mg
2	Sodium acetate (10 mM)	1 ml

8. TE buffer (pH:7.4) -100 ml		
1	Tris-Cl (pH-7.4)	1 ml
2	EDTA (pH 8)	0.2 ml
3	Water	98.8 ml

Phenol: Chloroform: IAA – Mix equilibrated phenol, chloroform and isoamyl alcohol in the ratio of 25:24:1 (v/v).

Appendix II: Agarose gel electrophoresis

a) Loading dye composition (6X)
1. 0.25 % Bromo phenol blue (BPB)
2. 40 % sucrose
3. Water to make up the volume (100 ml)

b) Ethidium bromide
10 mg/ml in distilled water and stored at 4^0C in dark bottle.

c) Preparation of 1.5 % Agarose gel (100 ml)
1. Agarose 1.5 g
2. 1X TAE 100 ml
3. EtBr (10mg/ml) 4 µl

d) 50X TAE composition
1. Tris base 242 g
2.Glacial acetic acid 57.1 ml
3. 0.5 M EDTA (pH 8.0) 100 ml
Total volume made up to 1000 ml with double distilled water

Appendix III: Denaturant Gradient Gel Electrophoresis (DGGE)

Polyacrylamide solution (100 ml) – Dissolve 37.5 g of acrylamide and 1 g of bisacrylamide in 80 ml distilled water and make up the volume to 100 ml with double distilled water.

Preparation of denaturant gradient gel			
Sl. No.	**Components**	**Solution A 45 % denaturant and 8 % gel**	**Solution B 60 % denaturant and 8 % gel**
1	Polyacrylamide	20.78 ml	20.78 ml
2	50X TAE	2 ml	2 ml
3	Formamide	18 ml	24 ml
4	Urea	18.9 g	25.2 g
Note: The final volume made up to 100 ml of solution A and solution B using nanopure water			

Preparation of 5 % staking gel (10 ml)		
Sl. No.	**Components**	**Volume (µl)**
1	Polyacrylamide	1298
2	50X TAE	200
3	20 % APS	80
4	TEMED	5
5	Nanopure water	8417

Preparation of silver staining solutions		
Sl. No.	**Solutions used**	**Reagents**
1	Fixer	100 ml ethanol and 5 ml glacial acetic acid
2	Wash	Deionised water
3	Impregnation solution	1.5 g $AgNO_3$ and 1 ml formaldehyde
4	Rinse	Deionised water
5	Developer solution	15 g NaOH and 2 ml formaldehyde
6	Stop solution	100 ml ethanol and 5 ml glacial acetic acid
Note: Make up the volume of each solution to 1000 ml by using nanopure water		

METAGENOMIC ANALYSIS OF PANCHAGAVYA

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ABSTRACT

Panchagavya is the blend of five ingredients obtained from cow. The present research focused mainly on studying the microbial succession at different stages of panchagavya making use of culture independent approach. The DNA isolation protocols for panchagavya samples drawn at different intervals, individual ingredients, phylloplane of soybean sprayed with panchagavya along with their respective control were standardized. Specific primers targeting variable region of 16S rRNA were used in PCR amplification. Using DGGE analysis the microbial community composition, diversity, richness, carrying capacity and dynamics present in these samples of panchagavya preparation besides, individual ingredients and phylloplane samples were studied. GCMS analysis of panchagavya revealed a total of 36 compounds present in panchagavya samples. Fifteen of these compounds were reported to have biological activities such as antibacterial, antifungal, antioxidants, antitumor and nematicidal activity while the activity of the remaining 21 is yet to be known. Sequence based comparative analysis between the three stages of panchagavya at phylum level showed that Firmicutes, Actinobacteria and Proteobacteria were higher in number in 30 days old panchagavya sample. Only Bacteroidetes was observed as the highest phylum in the panchagavya sample drawn on 14^{h} day. Bacteria belonging to unassigned genus were higher. Genera such as; *Megasphaera, Lactobacillus, Bulleidia* and *Rumminococcus* were observed in higher percentage in 30^{th} day panchagavya sample as compared to 14^{th} and 21^{st} days samples. From the study it is evident that panchagavya, ingredients and phylloplane contains specific microbial community with high diversity, high carrying capacity and even distribution of species showing medium level of dynamics between the stages of panchagavya. Panchagavya sample of 21 days old appeared to have more microbial diversity than 30^{th} day old panchagavya sample.