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Bacterial Succession During Panchagavya Making as Revealed by DGGE Analysis

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Panchagavya is the blend of five ingredients obtained from cow. Making of panchagavya is a microbial process that possibly involves microbial succession. Using culture independent, molecular fingerprinting technique (Denaturing Gradient Gel Electrophoresis), the bacterial community composition, diversity, richness, carrying capacity and population dynamics present at different stages of panchagavya preparations were studied. Panchagavya samples drawn on 15th day exhibited the highest bacterial species diversity while the least diversity was observed in 30 days old sample. Functional organization showed that in all samples, 80 to 95 per cent of bacterial populations were represented only for 20 bacterial species. The average change in bacterial diversity was 17.89 per cent which indicated a medium level of bacterial dynamics present at all the five consecutive stages of panchagavya making. The DGGE analysis of panchagavya clearly indicated microbial succession and high microbial diversity which was functionally even at all the stages of its making.

Key words: Panchagavya, metagenomics, diversity, denaturing gradient gel electrophoresis.

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, microflora, quality of water, food and fodder. Therefore, the current global scenario firmly emphasizes the need to adopt eco-friendly agricultural practices for sustainable and quality food production. Organic farming envisages the comprehensive management approach to improve the soil health, eco-system of the region and the quality of product. A number of indigenous organic sources of nutrients like organic manures, enriched manures, vermicompost, green manures and also liquid organic manures such as *Beejamruth*, *Jeevamruth*, *Amruthpani* and *Panchagavya* etc., are commonly used by the farmers engaged in organic food production. Of these liquid organic manures, "Panchagavya" is more widely used as a traditional practice to safeguard plants, soil microorganisms and to increase plant productivity. Panchagavya has shown beneficial effects on a variety of crops[1]. In Sanskrit, Panchagavya means the blend of five products obtained from cow. The three direct constituents are dung, urine and milk while the two derived products used are curd and ghee. They are a rich source of both nutrients and microorganisms. When the above five products of the cow are suitably mixed and used, these have miraculous positive influence on living organisms[2]. Existing scientific knowledge about panchagavya clearly suggests 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. Although there is mention of

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the beneficial effects of cow products[3] used in panchagavya making the microbial process involved in its preparation appears to have not been studied since ancient times. This study employing a culture independent technique is an attempt to understand the microbial process involved in panchagavya making, as culture dependent techniques are inherently inadequate to reveal greater microbial diversity[4]. Use of specific primers to amplify bacterial ribosomal genes from the metagenome and analyze them using DGGE to understand microbial diversity is known to be very useful culture independent approach. Data generated from such methods when analysed using suitable statistical tools can reveal microbial diversity in terms of bacterial richness, diversity, distribution of species (evenness) and functional organization of a species within a sample. All these were attempted in the present study and the shift in bacterial community of panchagavya over a time period was studied.

MATERIAL AND METHODS

Ingredients of panchagavya

Five ingredients essential for panchagavya preparation namely; cow dung, cow urine, cow milk, curd and ghee were freshly collected and used. Cow dung, urine and milk were freshly collected from the dairy unit of Institute of Organic Farming (IOF), University of Agricultural Sciences, Dharwad (UASD). The curd was prepared using cow milk obtained from the same source. 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 and cooled milk was poured under aseptic conditions. To this milk, 25 ml starter culture of curd from previous batches was added, stirred and kept at room temperature for eight hours. Fresh ghee made from cow milk was procured from the commercial outlets of Karnataka Milk Federation (KMF) and used in making panchagavya.

Preparation of panchagavya under laboratory conditions

In the present study, panchagavya was prepared using a protocol developed previously[2]. The ingredients quantity was reduced to 1/10th volume of original protocol with minor modifications. Accordingly, 600 g of fresh cow dung and 100 g of ghee were mixed thoroughly and kept for two days. Then, on the 3rd day, 400 ml of fresh cow urine and one litre of water were added, stirred and left for another 12 days. Finally, on the 15th day other ingredients like 300 ml of fresh sugarcane juice, 200 ml of fresh cow milk, 200 ml of freshly prepared curd (eight hr culture), 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 in clock and anti-clock direction twice a day. The preparation was carried out in five litre glass bottles under ambient conditions.

Extraction of microbial community DNA from panchagavya

Before extracting DNA from Panchagavya, the sample was thoroughly shaken to mix the content. Direct method of DNA extraction[5] was used for isolating microbial community DNA from panchagavya samples starting from 1st day to 30th day at a regular interval of seven days.

Metagenomic DNA was extracted using the protocol[6] with some modifications[7]. Panchagavya sample was filtered through four layered muslin cloth. Five ml of filtered panchagavya sample was added to 15 ml of the DNA extraction buffer [100 mM Tris HCl (pH 8.0), 120 mM EDTA (pH 8.0), 100 mM $CaCl_2$, 100 mM sodium phosphate buffer (pH 8.0), 1.5 M NaCl, 1 % hexadecylmethylammonium bromide (CTAB)] and incubated for 30 minute at 37 °C with rotary agitation at 120 rpm. To the content 50 µl of Proteinase K (20 mg/ml) was added and incubated for 1 h at 37 ºC under shaking condition at 150 rpm. To this mixture two ml of 20 % SDS solution was added and incubated at 65 ºC for two h in a water bath with gentle, intermittent mixing every 20 min. Further, the contents were centrifuged for 10 min, at 10000 rpm and the supernatant was collected separately. The collected supernatant was mixed with an equal volume of phenol: chloroform: isoamylalcohol (25:24:1 v/v) solution. All the contents were agitated gently for 30 min and centrifuged for 10 min at 10000 rpm to collect the supernatant. This step was repeated, however with equal volume of chloroform: isoamylalcohol solution (24:1 v/v), and the supernatant was

collected. The DNA in supernatant was precipitated by adding 1/6th volume of isopropanol and incubated for one h at room temperature. The mixture was subsequently centrifuged for 20 min at 10000 rpm and the precipitated DNA pellet was washed with 70 % ethanol. Finally, pellet obtained was dissolved in 50 ìl of sterile $T_{10}E_1$ buffer and stored at -20 ºC until further use.

PCR Amplification of 16S rRNA gene using universal primer pair

Purified DNA samples were taken for PCR amplification. Hypervariable region (V3) of 16S rDNA was amplified using PRBA338 with GC clamp and PRUN518 primers[8]. The reaction mixture was prepared for a final volume of 10 ìl, 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 $MgCl_2$ (GeNei, India) and one unit of Taq DNA polymerase (GeNei, India). The PCR was performed in automated thermal cycler (Eppendorf master cycler, Germany) with the following PCR programme; initial denaturation for seven minutes at 95 °C followed by denaturation at 94 ºC for 45 seconds, annealing at 55 ºC for 45 seconds and primer extension for 45 seconds, for a number of cycles 32, followed by 10 minutes of final extension at 72 ºC. After completion of PCR, amplified products were analysed using 1 % agarose gel.

Denaturing Gradient Gel Electrophoresis (DGGE) analysis

PCR reaction was carried out for all the five samples of panchagavya taken at respective intervals, The PCR products were subjected to DGGE analysis by following the protocol available[9]. After achieving separation on gels, the gels were carefully removed and stained using silver stain[10]. The stained gel was dried sufficiently and was analyzed using SynGene Gene Tools. Bands were scored using this tool, by giving the lowest score to the least intense band in the gel. Based on the score the data obtained was used to calculate Sorenson's similarity coefficient, Shannon's diversity index[11], Range weighted richness[12], Pielou's evenness index[13], Pareto Lorenz curve[14] and Moving window analysis[15]. These derived values were used to analyze the similarity, diversity, carrying capacity, species evenness, functional organization of species and microbial dynamics present during the different stages of panchagavya preparation.

RESULTS

Metagenomic DNA isolation and PCR amplification from panchagavya samples

The metagenomic DNA extracted using this protocol was of 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/ìl with a purity ratio of 1.78-1.87 at 260/280 as measured using Nano Drop ND 1000 Spectrophotometer (Fig.1). The primer used could amplify the targeted site with an expected size of 180 bp. There was no amplification in the negative control indicating no contamination in the PCR components and that the used primers targeted the exact region of 16S rRNA.

Analysis of DGGE profile

The DGGE profile of all the samples studied were distinctly different and were reproducible. Several bands, each indicating an operational taxonomic unit (OTU) was observed in all the samples (Fig. 2). While most OTU's were of medium to low intensity, few were distinctly prominent. Some OTU's unique to each stage of panchagavya were also observed. The qualitative (presence and absence) and quantitative (band

Table 1. Bacterial richness, evenness and diversity at developmental stages of panchagavya calculated based on DGGE fingerprint.

Stages of panchagavya	Number of OUT's	Range weighted richness	Pielou's evenness index	Shannon diversity index
3rd Day sample	27	209.58	0.84	2.74
7th Day sample	28	228.92	0.84	2.80
15th Day sample	28	181.88	0.88	2.96
21st Day sample	23	114.79	0.89	282
30th Day sample	23	122.11	0.74	2.35

Table 2. Sorenson's pair-wise similarity index for microbial community of panchagavya sample at different stages

Stages of panchagavya	3rd Day sample	7th Day sample	14th Day sample	21st Day sample	30th Day sample
3rd Day sample	1.00				
7th Day sample	0.55	1.00			
14th Day sample	0.17	0.28	1.00		
21st Day sample	0.24	0.36	0.65	1.00	
30th Day sample	0.21	0.23	0.14	0.15	1.00

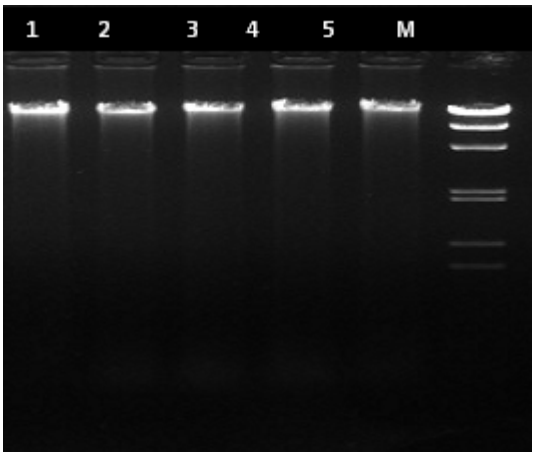

Fig. 1. Genomic DNA isolated from different stages of panchagavya, 1 – 3rd day, 2 – 7th day, 3 – 14th day, 4 – 21st day, 5 – 30th day old panchagavya samples.

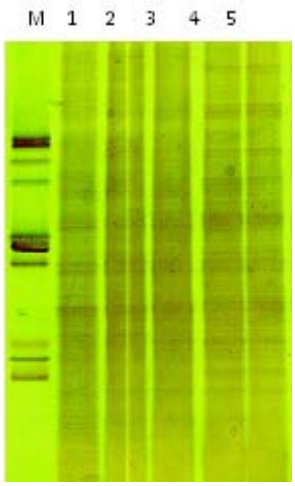

Fig. 2. PCR-DGGE profile of panchagavya bacterial species samples drawn on 1- 3rd day, 2- 7th day, 3- 15th day, 4- 21st day and 5- 30th day old panchagavya samples. 16S rDNA amplified by PRBA338GC and PRUN518 primers was separated in 12 % polyacrylamide gel containing 30%-70% denaturant and silver stained

intensity) data of DGGE profile was converted into numerical values using Gene tools (Syngene) software. This numerical data was further processed to estimate species richness, diversity and their distribution pattern.

Shannon diversity indices were calculated for the bacteria present in panchagavya sampled on 3rd, 7th, 15th, 21st and 30th day of their preparation based on the DGGE analysis. It was evident from the values obtained (Table 1) that the diversity of bacteria increased from 3rd day (2.79) to 15th day (2.96) during the process of panchagavya making, there after declined on 21st day (2.82) until 30th day (2.35). Range-Weighted Richness (Rr) values were estimated on a denaturing gradient of 30-70 per cent range (Table 1). Numbers of bands or OTUs under each sample were critically analyzed to elucidate species richness in each respective sample. The range weighted richness values were found to be higher (all were more than 30) for all the panchagavya samples drawn at different intervals. Sorenson's similarity index of panchagavya showed that the sample drawn on 15th and 21st day shared the highest microbial similarity of 65 per cent. This was followed by the similarity (55 %) shared between samples drawn on 3rd and 7th day. However, samples drawn on 15th and 30th days shared the lowest similarity of 14 per cent (Table 2). Pielou's evenness index values for population of bacteria were found to be highly even in most of the samples except for the sample drawn on 30th day. Pareto Lorenz (PL) curve of panchagavya samples showed that more than 80 per cent of bacterial populations observed belonging to only 20 per cent of species which were functionally more organized (Fig.3). Microbial community dynamics was 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 panchagavya preparation was studied using moving window analysis (Fig.4). The bacterial community dynamics was found to be higher during the early days of panchagavya preparation but after about 15 days it tended to be more stabilized, implying that this could be the climax microbial community structure. The overall shift in bacterial community across all the stages of panchagavya was 17.9 per cent (Fig.4).

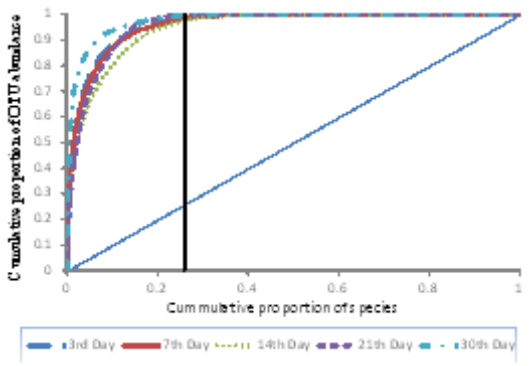

Fig. 3. Pareto–Lorenz (PL) curves derived from the DGGE pattern of five different stages of panchagavya samples. Large number of individuals belongs to only few species reflecting these panchagavya bacteria are highly functionally organised

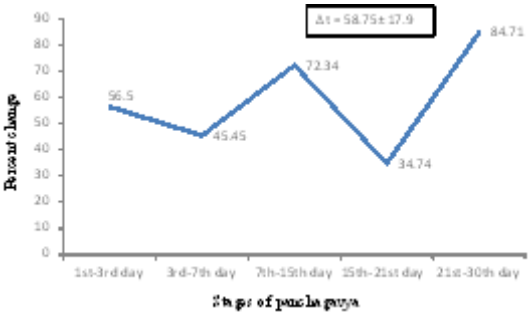

Fig. 4. Shift in bacterial community composition during developmental stages of panchagavya analysed by moving window analysis. During early stages, the community was more similar, but it becomes more dissimilar during later stages. Overall change in bacterial community structure during developmental stages of panchagavya (Δt) was 17.9 represent medium level of dynamic present in panchagavya stages.

DISCUSSION

The conventional techniques involving culture dependent methods such as the cultivation of microbes, phenotypic characterization and PCR-based approaches often are met with a limitation of accessing only one to five per cent of bacterial diversity from environmental samples[16]. The present study aimed at accessing the metagenome of panchagavya to understand bacterial diversity using DGGE a culture independent technique. It is probably for the first time an attempt is being made to understand the microbial diversity employing culture independent approach in panchagavya, which is a traditional Indian liquid organic plant growth promoter.

Isolation of metagenomic DNA from different stages of panchagavya

Accessing environmental microbial consortium, including DNA extraction and purification[17] has been challenging task in metagenomic analysis. Further, extraction of nucleic acids from panchagavya sample has the practical difficulty, especially because all the species present in the individual ingredient of panchagavya may not have the same sensitivity to the lytic agents and extraction buffers due to differences in their cell wall organization[18]. Difficulty is experienced in obtaining high quality metagenomic DNA from ecological samples such as, soil, organic matter, compost, dung and panchagavya because of the humic substances present in them. The humic substances are chemically, highly complex and are difficult to remove, unless additional, laborious and time intensive treatments are employed to obtain good DNA from them for downstream molecular methods like PCR and restriction digestion[19,20]. In the present study the protocol for isolation of total DNA from panchagavya at various stages of preparation was modified and standardized. Addition of 100 mM $CaCl_2$, 120 mM EDTA and two ml of 20 % SDS in the DNA extraction buffer during DNA isolation was useful modifications to maximize the DNA yield and to minimize the contamination by inhibitor substances. The yield of DNA isolated using this modified protocol was in the range of 1150- 1635 ng/ìl, which was of high quality and purity. This type of high quality metagenomic DNA is considered a prerequisite for downstream process[21]. Further, the DNA obtained was diluted

and used for PCR amplification which could also be attributed to high quality of metagenome isolated.

PCR amplification of isolated metagenomic DNA

In prokaryotes, the 16S ribosomal RNA (rRNA) genes are the most conserved and occur at least in one copy per genome[22]. The universality of the genes makes them an ideal target for phylogenetic studies and taxonomic classification. Universal primers targeting 16S rRNA genes[23] were employed in the present study and they were found very useful to obtain amplicons of 180 bp as expected. Primers targeting V3 regions have been used previously for species identification[24]. Earlier experiments successfully used primers targeting V3 region of 16S rDNA and further analyzed them using DGGE and pyrosequencing[25]. This variant region in 16S rDNA has provided sufficient phylogenetic information about bacteria in the samples[26, 27]. Therefore, in the present study the primers set used (PRBA338-PRUN518) targeted the V3 region of 16S rDNA[8]. The choice of the primers used and the reaction conditions for PCR amplification of 16S rDNA variable region in this study were appropriate to obtain desired amplicons of 16S rDNA from metagenomes of panchagavya.

Analysis of DGGE profile

DGGE is one of the most well established molecular tools in microbiology[4, 9]. This can be used to identify bacterial species from natural environment on the basis of variable regions present in their 16S rDNA genes [28]. Each band that appears has a very high versatility, reproducibility and is considered as a separate OTU or species[29]. Hence, 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 as done in this study. These data provide an insight into microbial community structure, population and dynamics which remain obscure by other culturing methods[29].

Diversity, Richness, Similarity and Evenness of bacterial population present in panchagavya samples

Shannon diversity index has been one of the most widely used parameters to access biodiversity; it measures the average degree of distribution of species of a given individual within a randomly chosen population. 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[11]). 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 15th day (2.96) followed by the sample drawn on 21st day (2.82) indicating that panchagavya samples drawn on 15th and 21st days were having more species richness and species abundance. This is likely to provide an insight into diverse microbial groups present in similar proportion and their relative distribution at given space and time. The lowest value of Shannon index (2.35) was observed with metagenome of panchagavya sample drawn on 30th day (Table 1). This possibly occurred as the bacterial species and their number might have decline from 21st day until 30th day.

In the preparation of panchagavya in this study ingredient at the starting included, 600 g of cow dung and 100 g of ghee along with 400 ml of cow urine and 1000 ml of water, Shannon diversity was lower during the early stages of panchagavya till 7th day possibly because of microflora represented mainly from cow dung. Other ingredients 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 on 15th day. The total microbial diversity from all ingredients was appeared to have contributed for higher Shannon value (2.96) on 15th day. These ingredients are either rich source of nutrient and or have inherently abundant bacterial flora which could have contributed to higher Shannon diversity in panchagavya samples on after 15th day. Cow dung has been rich source of nutrient and bacteria[30, 31] thus; diverse microfloras in panchagavya after 15th day might have come from the ingredients such as dung, milk and curd. 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 15th day (2.96) to 30th day (2.35) bacteria. 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 species richness in the metagenomic DNA was calculated based on the number of bands (OTUs) present per sample between 30-70 per cent denaturing gradient. Critically analyzed bands were used to calculate species richness and expressed as range weighted richness (Rr). The Rr values were observed to be more than 30, which implied that panchagavya is a typical and very habitable environment with broad carrying capacity, having high microbial diversity. Hence, it could be called as high range weighted richness sample[12]

Sorenson's similarity index was used to measure similarity in the bacterial composition between samples. A similarity index of 100 per cent indicated that DGGE profiles were identical while completely different profiles had a value of zero per cent. Similarly, Sorenson's similarity index showed that panchagavya of 15th and 21st days 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 3rd and 7th days which sheared 55 per cent similarity of bacteria. However, samples drawn on 15th and 30th days shared only 14 per cent of species similarity (Table 2). Panchagavya sample of 3rd and 7th day contained only cow dung, cow urine, ghee and water so the microflora of dung and ghee were possibly represented. The highest similarity was thus observed between those two samples. Similarly, 15th and 21st day have shown highest similarity values because, on 15th day added other ingredients as mentioned in panchagavya preparation were added. Their native bacteria and nutrients probably sustained the diversity until 21st day. Small fluctuations and drops in bacterial populations observed on 30th day panchagavya could be caused due to changes in environment of panchagavya especially nutrients, aeration and accumulation of metabolites. Bacteria capable of surviving in that condition only prevailed in 30th day panchagavya sample. The Pareto Lorenz curve indicated that a specialized community of bacteria were present in 30 days old panchagavya. It indicated that a small amount of the species was dominant and all the others were present in low numbers, with a large difference between the two groups. Also the changed redox potential in the system could have shift caused in the bacterial species.

Pielou's evenness values of panchagavya at all the stages were observed to be more than 0.75 per cent (Table 1). Results showed that species were more evenly distributed at all the stages of panchagavya. Pielou's evenness index value of one and closer to it indicated highly even distribution of species in a sample and index value of zero and closer values indicated highly uneven distribution of species in a sample[13]. It was very clear that highly even bacterial species distribution occurred at all stages of panchagavya preparation.

Functional organization of bacterial species present in panchagavya using Pareto Lorenz (PL) curve

Pareto Lorenz curves for panchagavya samples drawn at different stages showed that the curve values for all the samples were more than 80 per cent on Y axis at a 20 per cent intercept on X-axis. This implied that more than 80 % bacterial population in panchagavya was belonging to only 20 bacterial species (Lorenz, 1905). It is likely that a specialized microbial community could exist in each sample[32]. As observed[12] in their study such microbial community could be functionally highly organized but fragile to external changes because disruption might require longer recovery time by the existing bacteria and to rebuild their favourable environment.

Bacterial community dynamics present at different stages of panchagavya

Dynamics of a microbial community in a sample is a measure of the average rate of change in parameter and degree of change between consecutive DGGE profiles of the same community over a fixed time interval[33]. Based on moving window analysis, the rate of change (D*t*) in parameter was calculated at all the consecutive stages of panchagavya at defined time interval. It was observed that the rate of change in parameters was 17.9 per cent. The values for overall per cent change ranged between 13 and 25 per cent. This situation was assumed to represent a medium level of bacterial population dynamics present at all stages of panchagavya. Further, it could imply that in panchagavya sample a new species can entered

into pre existing bacterial community but cannot interfere with the functionality of the pre-existing population as observed[34] in this study.

CONCLUSION

This is possibly the first research report on standardizing protocols for obtaining high quality metagenomic DNA from panchagavya. Very high bacterial diversity in panchagavya samples at different intervals was observed. The bacterial diversity, richness and evenness at all the stages were found to be high while it was the highest on 15th day of panchagavya preparation. This possibly contributed from other ingredients supplemented on 15th day. A medium level of bacterial population dynamics was observed at all stages of panchagavya preparation possibly because of alteration in the environment. This medium level of change in bacterial population did not affect the total functionality of bacteria present in panchagavya. This could be attributed to its beneficial effect when used as an organic amendment.

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