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A Preliminary Study to Understand the Microbial Composition of *Fresh Panchagavya* Preparation

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Abstract: Fecal Microbial Transplant (FMT) has been emerging as a prominent therapeutics in the modern science to modulate and correct the dysbiotic state of gut microbiome that leads to various metabolic and gastro-enteric diseases. A similar concept called as "panchagavya prasahan" exists in Ayurveda. This technique unlike the FMT involves the use of Cive by- products of cow viz. Cow dung, urine, milk, curd, ghee mixed in a copper vessel. In thisstudy we assess the microbial composition of this preparation before and after the mixing in the copper vessel. We used the 16S rRNA gene based amplicon sequencing approach to understand the bacterial composition in the *panchagavya* preparation. We observed signiCicant decrease in the abundance of genera *Pseudomonas* and *Streptococcus* ($p<0.05$) and signiCicant increase in abundance of genus *Lactococcus* after processing ($p<0.05$) the preparation in the copper vessel. The former two are known to belong to the pathogenic groups, while *Lactococcus* is known for its probiotic attributes and is widely used in the probiotic preparations. This certainly gives some insights into the potential beneCits of *Panchagavya*. Thus, '*Panchagavya prashan*' being a well-established technique in ayurveda may beneCit by improving the gut microbial balance and understanding the mechanism through the gut microbiome perspective is necessary.

Keywords: gut-microbiota; panchagavya; genome; probiotics; 16S rRNA; gut

Introduction

Fecal Microbial transplant (FMT) is now-a-days considered as a well-established technique used to treat gastro-intestinal diseases worldwide, especially the *Clostridium dif9icile* infection (CDI) in the western world [Ref]. Faecal microbiota transplantation (FMT) refers to the infusion of a faecal suspension from a healthy person into the gastrointestinal (GI) tract of another person to cure a speciCic disease [Ref]. It is a procedure in which fecal matter, or stool, is collected from a tested donor, mixed with a saline or other solution, strained, and placed in a patient, by colonoscopy, endoscopy, sigmoidoscopy, or enema [Ref]. Faecal transplant was Cirst documented in 4th century China, known as "yellow soup"[Ref]. However, there is a similar concept in ayurveda "*panchagavya prasahan*", which is being practiced since ancient times, but have not been explored using modern scientiCic tools. *Panchagavya* is a system of medicine just like as homeopathy, allopathy or naturopathy [1,2]. The ancient ayurvedic literature (*Charak Samhita*, *Sushrut*, *Gada nigrah*) suggests a number of pharmacological applications of the substances obtained from *Panchgavya*. Religious ritual of practice of '*Panchagavya prashan'* has been in existence for ages in our country. This practice was expected to deliver a person from all the sins (*papma*in Sanskrit), which is also a synonym for the word 'disease'. This ritual is practiced once every year during July and August at which time the Vaccinia viraemia is at its peak in cows[3]. A systematic work needs to be carried out on chemical nature, biological activity, microbiology and pharmaceutical aspects and mechanism of bioactive compounds in *panchagavya*. In the recent past due emphasis has not been given to this therapy by scientiCic community. Our aim is to decode the basis of this practice in modern scientiCic language.

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Panchagavya involves the use of Cive by-products of cow viz. Cow dung, urine, milk, curd, ghee mixed in a copper vessel [3,4,6]. Copper vessel is used because it iscapable of detoxifying and destroying disease causing bacteria (potential pathogens) through ionization [3]. Fresh urine being acidic in nature is antitoxic and helps to attenuate bacteria [3]. Fresh milk and curd are also probiotic in nature and act as neutral nutritional medium to protect attenuated organisms [3].

Ghee helps in enteric coating the organisms thereby preventing their destruction by gastric acid. Cow dung is expected to harbor all the enteric organisms. Since it's a season when the vaccinia is rampant in cows it is expected the vaccinia virus is secreted in to all the secretions and bodily excretions of cow. Previous studies suggest that when the dung is treated with acidic urine especially in a copper container, which ionizes, the organisms undergo deCinite attenuation [7]. When these attenuated organisms reach the intestinal villiand Payer's patches, they stimulate activate general immunity and as well the speciCic immunity [4,5]. Secondly, there will be new bacterial colonies of cow origin introduced intothe gut. This is also expected to change the gut microbiota composition and thereby causingdeCinite changes that may be desirable in certain medical conditions, e.g. IBS, Ulcerative colitis, diabetes mellitus, obesity etc. Diseases have been traditionally studied under a paradigm of "one microbe, one disease." However, a new understanding is emerging on howdisease phenotypes are actually a result of complex interactions between bacteria, viruses, and eukaryotes, as well as their interactions with the host or with certain drugs [8]. Virulence of some eukaryotes is, for instance, linked to the presence of certain bacteria, such as in the case of *E. histolytica* and *E. coli* or *S. dysenteriae* [8]. Interestingly, studies haveproven that the susceptibility of the host to viral infections is conditioned by the particular conCiguration of the microbiota, whereas herpesvirus infection can confer resistance to certain bacterial infections [8,9]. As a clear correlation has been observed between many diseases and dysbiosis, restoring a healthy microbial community by administration of *panchagavya* can be proven to be a valuable tool in the treatment of these diseases.

Further, use of antibiotics has certainly proven to be ineffective in treating many gastrointestinal diseases [10]. Considering the rapid emergence of these lifestyle associateddisease, there is an urge of developing novel tools to modulate the gut microbiota and eventually improve the health [11]. *Panchagavya* prashan being a well-establishedtechnique in ayurveda may beneCit by improving the gut microbial balance and may also improve the immunological proCile of the individual. Thus, understanding the mechanism through the gut microbiome perspective is necessary. In this study, we present a preliminary investigation on the microbial composition of the fresh '*panchagavya*' preparation and the effect of process of using copper vessel on the observed microbial diversity. This study provides a basis for future investigations on '*panchagavya prashan*' and to understand its inCluence on the gut microbial composition and eventually on the human health.

Material and Methods

Panchagavya preparation

Protocol for Fresh Panchagavya Preparation

The Cive or *panch* ingredients of Panchagavya are cow urine, fresh cow dung, cow milk, cowcurd and cow ghee.

The products with their quantities are:-

Fresh cow dung: ½, Cow urine: 1, Cow milk: 7, Cow curd: 1, Cow ghee: 1.Method of Preparation:

Step 1: Mix fresh cow dung and cow urine thoroughly and keep it for 30 minutes, Cilter this mixture using muslin cloth transfer the Ciltrate to copper vessel.

Step 2: Add cow milk and cow curd to the Ciltrate and mix well for 10-15 minutes.

Step 3: To the above mixture add liqueCied cow ghee and centrifuge the mixture at lowestpossible rpm for 10 minutes.

Step 4: Transfer the Fresh Panchagavya mixture to a sterile container.

DNA Extraction and PCR Ampli5ication

Aliquots of ~1 gram of freshly prepared *panchagavya* preparation (DK1) and panchagavya preparation after processing in the copper vessel (DK2) were taken for total DNA extractionusing the

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QiAmp mini stool DNA extraction kit (Qiagen, USA) as per the manufacturer's protocol. The DNA extraction was done in replicates (in duplicate) and all the replicates were then pooled together in 1:1 ratio. The total DNA extracted DNA was then quantiCied using the NanoDrop spectrophotometer ND-1000 (Thermo ScientiCics, USA) and stored at -20° C until further use. The DNA samples were used further for the 16S rRNA basedamplicon library for high throughput sequencing. BrieCly, the V3 region of the 16S rRNA gene was ampliCied using the region speciCic bacterial universal primers: forward primer 341F (5′-CCTACGGGAGGCAGCAG-3′) and reverse primer 518 R (5′-ATTACCGCGGCTGCTGG-3′; Bartram *et al.*, 2011) and methodology as described earlier[Bhute *et al.*, 2016].

Ion Torrent Based Amplicon Sequencing

The library generation and sequencing was carried out as previously described by Bhute *et al.*, 2016. BrieCly, PCR products were puriCied using Agencourt AMPure XP DNA puriCicationBead (Beckman Coulter, USA). The puriCied products were end repaired and ligated with speciCic barcode adaptor as explained in IonXpress™ Plus gDNA Fragment Library Preparation user guide. Fragment size distribution and molar concentrations of amplicon were assessed on a Bioanalyzer 2100 (Agilent Technologies, USA) using High Sensitivity DNA Analysis Kit as per manufacturer's instructions. Emulsion PCR was carried out on diluted and pooled amplicon using the Ion One Touch™ 200 Template Kit v2 DL (Life Technologies). The sequencing of the amplicon library was carried out on Ion Torrentsequencing platform using the Ion 316 chip with Ion Sequencing 200 kit, as per the manufacturer's instructions (Life Technologies, USA).

Bacterial Diversity Analysis

The raw reads obtained from the sequencing run were assessed for quality using theFASTQC tool [12]. The adapter sequences were trimmed and the quality based trimming of the reads was done using Mothur [13]. Further, these quality processed sequences were used for OTU (Operational Taxonomic Sequencing) clustering using the SILVA database. The representative set of these sequences per OTU cluster were then picked and assigned taxonomy using the same SILVA taxonomy database using QIIME (Quantitative Insights In Microbial Ecology) pipeline [14]. The BIOM Cile thus generated using the standard QIIME pipeline was then further used to carry out composition and statistical analysis using the tools such as STAMP [15]. Appropriate statistical tests such as Wilcoxon signed-rank matchpaired t-test were used to determine the statistical signiCicance between the diversity in preand post processing *panchagavya* samples.

Results

Sequencing and Bacterial Composition of the Panchagavya Preparation

The high throughput sequencing carried out using Ion Torrent single end sequencing technology generated 14,642 reads for sample DK1, while 13,094 reads for sample DK2. The quality Ciltering using Mothur (>QV20) was employed to get quality sequences to be used for further analysis. It was observed that the more than 50 % erroneous reads were removed, yielding around 5.5K reads per sample. The OTU clustering using QIIME, using SILVA databases (SILVA_99) reference dataset, yielded 1,694 OTUs for DK1 and 1,756 OTUsfor DK2 sample.

The analysis carried out to understand the overall bacterial composition of the*panchagavya* preparation revealed that the bacterial diversity was dominated by phyla *Firmicutes* (mean=81.94%), followed by *Proteobacteria* (mean=9.27%) and *Bacteroidetes* (mean=6.82%). Other rare taxa such as *Fibrobacteres*, *Actinobacteria*, *Cyanobacteria* and *Saccharibacteria* were also observed (<0.2%) in the *panchagavya* preparation in both pre and post processing in the copper vessel (See Figure 1). Further we also analysed the diversity at the deeper taxonomic level i.e. at OTU (Operational Taxonomic Unit) level. It was observed that the taxa *Ruminococcaceae* UCG-005 and *Ruminococcaceae* UCG-010 werethe most dominant taxa (mean=17.19%) seen followed by *Streptococcus* (mean=0.56%) *Christensenellaceae*

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R-7 group (mean=0.48%), *Pseudomonas* and *Lactococcus* (mean=0.42%and 0.40% respectively) along with other rare taxa (See Figure 2).

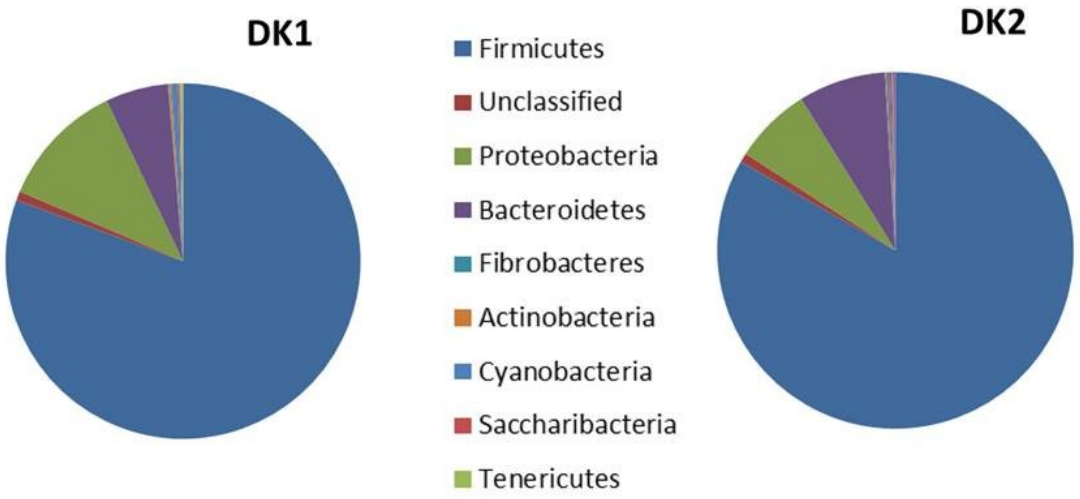

Figure 1. Phylum level distribution of bacterial diversity of *Panchagavya* samples pre(DK1) and post (DK2) processing in the copper vessel.

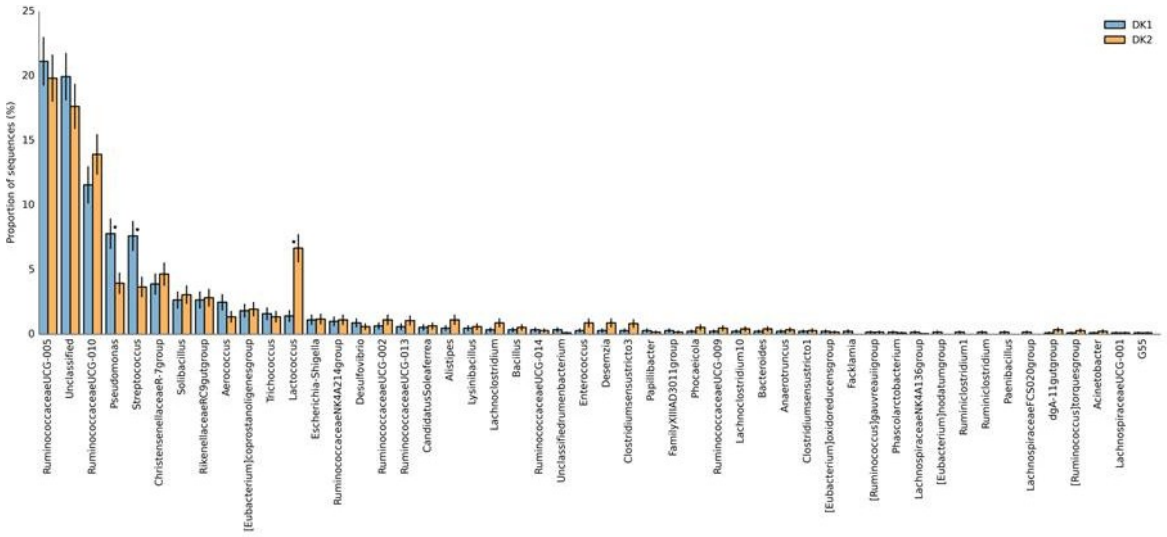

Figure 2. Differences in the bacterial diversity abundance between pre and post processingsamples. The asterisk (*) mark indicates taxa with statistically significantly differences between two samples using Wilcoxon signed-rank match paired t-test.

Differences in the Diversity between Pre and Post Processing

We investigated the differences in the bacterial diversity between pre- (DK1) and post- (DK2) processing samples of *panchagavya*. Our analysis showed that the overall bacterial diversity underwent few compositional changes in the terms of the abundance of genera, out of which 3 genera were observed to be signiCicantly different ($p<0.05$, Wilcoxon signed-rank match paired t-test) between two samples. SpeciCically, the OTU belonging to *Pseudomonas* and *Streptococcus* (See Figure 2) were observed to be decreased signiCicantly in the post processing samples (DK2) as compared to the post processing samples (DK1). Further it was observed that the abundance of genus *Lactococcus* increases signiCicantly in the DK2 sample as compared to the DK1 sample (See Figure 2). Although not signiCicant, however marginal differences were observed in the abundance of other genera such as *Christensenellaceae* R-7 group and *Ruminococcaceae* UCG-010 (See Figure 2).

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Discussion

The preliminary study of the bacterial diversity of the *Panchagavya* preparation revealed a highly diverse bacterial composition. Our investigation further reveals differences in the bacterial diversity in the *Panchagavya* preparation after processing it in the copper vessel.

We used the high throughput sequencing technology to decipher the bacterial composition of the *Panchagavya* sample, which is known to be a Cive component mixtures from obtained from the cow. Our analysis revealed that the traditional Ayurvedic preparation possess a diverse group of bacterial members with few highly dominating taxa such as *Firmicutes*, *Proteobacteria* and *Bacteroidetes* at Phylum level, while was seen dominated by taxa *Ruminococcaceae* UCG-010 at lower classiCication level. Previous reports suggest that these bacterial taxa constitute the majority of the gut microbial composition of ruminant mammals [16,17]. As described earlier, the *Panchagavya* preparation also consists of the fresh milk and curd, which are known to be probiotic in nature, as they harbour beneCicial bacteria such as *Bi9idobacterium*, *Lactobacillus* and *Lactococcus* [18]. Although, the former two genera were observed in less abundance, OTUs belonging to genera *Lactococcus* were observed to be among one of the dominant taxa in the *Panchagavya* preparation.

The comparative analysis to understand the effect of processing the fresh panchagavya preparation in the copper vessel, revealed signiCicant differences in the abundances of few speciCic bacterial genera such as *Streptococcus*, *Pseudomanas* and *Lactococcus*. Many of the opportunistic human pathogens are reported to belong to *Streptococcus* and *Pseudomonas* genera. The abundance of these genera was observed to bedeclined significantly in the post processing sample as compared to the fresh *panchagavya* samples. These observations can be attributed to the effect of processing in the copper vessels, however further detailed investigations need to be carried out to Cind the mechanisms of the observed dynamics.

The sample size (Biological replicates) is one of the key limitations of the study, as increase in number of replicates will conCirm these observations with higher coincidence values. However, this study serves as a preliminary 'proof of concept' investigation which atleast provides the basic bacterial diversity proCile of the Ayurvedic '*Panchagavya*' preparation. This study also makes an attempt to understand the effect of processing the fresh '*Panchagavya*' sample in a copper vessel, which is a standard technique followed in Ayurveda. Though the effect is observed through the microbiome perspective, it at least provides important leads that there is a possibility of selective enrichment or reduction of speciCic bacterial members after the processing. Also, to the best of our knowledge this is the Cirst ever attempt made to understand the microbial diversity of the *Panchagavya* preparation, the use of which in Ayurvedic therapeutics is very well established. Hence the present study is a step towards such fundamental concepts and treatments in Ayurveda, using the modern molecular techniques.

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