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RESEARCH ARTICLE

ISOLATION OF MICRO-ORGANISMS FROM COMPONENTS OF PANCHAGAVYA

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A B S T R A C T

Panchagavya or Panchagavya gritha (PG) finds mention in many ancient texts in the scripts of Vedas and in texts related to the ancient Indian system of medicine Ayurveda. When literally translated from sanskrit, “Pancha” means Five and “Gavya” means substance or ingredient. In general, the term PG is used to signify the blend of five ingredients obtained from the cow namely urine (CU), dung (CD), milk (M), ghee (clarified butter, G) and curd (C). Ayurveda has mentioned that the five individual constituents of PG possess medicinal properties and can be used singly or in combination for treatment of different human ailments. On the other hand, a lot of criticism has been afforded to “cow therapy” by the western world. In this study, we decided to evaluate the microbial composition of the various components of PG and the effect of mixing them together. Our study indicates that CD and CU are heavy with a variety of micro-organisms, primarily yeast and gram positive and negative bacteria. Sequencing revealed the presence of *Acinetobacter, Klebsiella, Bacillus, Escherichia, Aeromonas, Lactococcus, Acinetobacter, Macrococcus, Aspergillus* and *Penicillium* genera from the colonies chosen from CD or CU separately or a combination of CD, CU, M, U and G. However, our study suggests that when all components of PG are combined together, they mitigate microbial growth in other components bringing down the total microbial load. Mixing them in a Cu vessel further brought down microbial load in PG to almost nil. Our study highlights the fact that PG made according to the recommendations of traditional literature retains only beneficial effects of cow therapy.

INTRODUCTION

Panchagavya (PG) is a widely used Ayurvedic formulation mentioned in various treatises of Ayurveda and has wide therapeutic indications. PG has been described in the Ayurvedic formulary of India and also included in the Ayurvedic Pharmacopoeia of India (API, 2007). PG contains five important substances obtained from cow, namely urine (CU), dung (CD), milk (M), ghee (G, clarified butter), and curd (C) (Jirankalgikar *et al*., 2013). Its anti-convulsant (Koneru *et al*., 2009) and hepatoprotective (Achliya *et al*., 2003) activities have also been evaluated and proven experimentally.

CU, an integral part of PG is mentioned as the most effective substance of animal origin with innumerable therapeutic value in ancient Indian Ayurvedic literature such as Charak Samhita and Sushruta Samhita (Dhama et la., 2005). Medicinal properties of CU such as bioenhancer, antibiotic, antifungal, and anticancer have been patented under US patent number 6,896,907 and 6,410,059 (Gosavi *et al*., 2011; Randhawa 2010). CU may also possess anti- potential probably due to its antioxidant properties (Gururaja *et al*., 2011, Sachdev *et al*., 2012). External application of CU on excision wound (EW) has shown to hasten the wound healing (Sanganal *et al*., 2014). Various studies have demonstrated that cow urine, as well as its distillate, has antibacterial and antifungal activity against various clinical strains of these pathogens (Jarald *et al*., 2008, Sathasivam *et al*., 2010). Thus although CU has been the subject of many studies, all the other components have not been studied either individually or in combination.

The usage of CD either directly or as a component of PG used in therapy has been severely criticised. Therefore we set out to examine the entire process of PG formulation. In this study, we formulated PG as per recommendations in traditional literature and isolated and characterized the microbes present in the components of PG namely CD, CU, M, G and C in various combinations and to see the effect on microbes present and total bacterial load.

MATERIALS AND METHODS

Materials

Fresh CU, CD, and M were collected from local cow farm using sterile containers and stored in refrigerator for further uses. Curd (C) and ghee (G, clarified butter) were obtained from the local market. All microbiological media were purchased from HiMedia Laboratories, Mumbai. Milk and curd used in these studies were non-pasteurised. CU used inthese studies was non-distilled.

Isolation of microorganisms from CD, CU, CD+CU, CD+CU+M+C or CD+CU+M+C+G

CD or CU indicate 1 gm of wet cow dung or 1 ml of cow urine added to 9 ml of sterile saline, respectively and then diluted serially with sterile saline upto 10^{-9}. CD+ CU (1:2) preparation was made by mixing 4 gm of CD was mized with 8 ml of CU and the mixture was then serially diluted with sterile saline. To prepare a CD+CD+M+C mixture, CD+ CU was first prepared as above. After incubation for 1 hr, 4 ml of curd and 8 ml of milk were added to CD+CU. This mixture was stirred with a sterile glass rod and the CD+CU+M+C mixture was then diluted serially with sterile saline. To prepare CD+CU+M+C+G mixture, CD+CU+M+C mixture was first prepared to which 1 ml of G (clarified butter) was added. The different media used were: MacConkey agar (MA), salmonella shigella agar (SSA), cetrimide agar, clostridial agar, soyabean casein digest agar (SCDA), potato dextrose agar (PDA) or nutrient agar were poured onto plates. Each set contained 1ml of each dilution, which was poured onto petri-dishes containing different media. Plates were incubated at 37 °C and colonies counted after 48 hrs of incubation. .

Maintenance of isolated organisms

Culture of organisms picked from MA plates was maintained on MA slants and that of SS was maintained on SS slants whereas colonies picked from SCDA plates were maintained on SCDA or NA slants. Fresh slants were prepared every 15 days.

Gram staining of isolated microorganisms

Isolated colonies on respective plates were picked by 2 mm loop and streaked on glass slides already having 100 uL of saline. A smear was prepared and dried in air. Gram staining was carried out using Crystal violet, Iodine and counter stained with Safranin O.

Sequencing of 16sRNA

Selected colonies derived either from CD or CD or CD+CU+M+C or CD+CU+M+C+G were sent on slants to GeneOmbio Technology, India for 16sRNA isolation and sequencing for identification of genera.

RESULTS

Microscopic characterization of microbes present in CD and CU

To characterize the microbial load present in CD and CU we cultured CD or CU diluents on soyabean casein digest agar (SCDA), an enriched medium to ensure that most microbes would grow. We saw a variety of microbes present on plates, from where slides were prepared and gram staining was carried out to study the morphology of microbes present in CD and CU. Examples are shown in Fig. 1. We observed a mixture of Gram positive and negative cocci (Fig. 1A). We also observed gram positive rods (Fig. 1 B). Fig 1C & D show gram negative rod shaped bacteria seen on the plates.

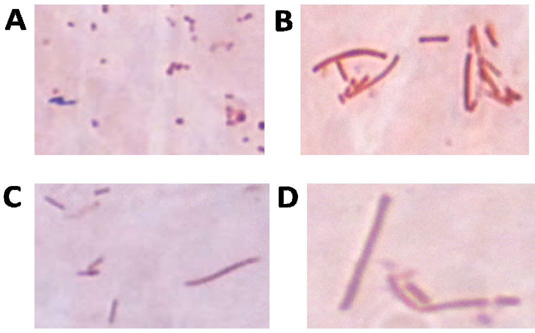

Fig. 1 Organism isolated from CD or CU or their combinations.

Microbial load from CD, CU and effect of addition of M+C on microbial load

Next we decided to estimate the microbial load present in CD and CU on NA plates to get an idea of the quantity of microbes that we were dealing with. We used dilution and gram staining to estimate the various species of microbes seen. As shown in Table 1, we saw sufficient colonies even at 10^{-9} dilutions of salmonella species, bacillus and gram negative coliform groups.

Table 1 Microbial load of micro-organisms isolated from CD + CU

Organism	Dilution			
	6-Oct	**10^{-7}**	**8-Oct**	**9-Oct**
SSA	>300	210	90	45
MA	>300	190	85	35
Bacillus species.	70	30	25	15

NA: Nutrient agar **CD**: Cow Dung, **CU:** Cow Urine

Since PG is a mix of 5 ingredients CD, CU, M, C and G, we decided to estimate the microbial load when M+C were added to the mixture of CD+CU. Table 2 shows that in the presence of M+C, no colonies of salmonella and bacillus were observed at dilution of 10^{-3}. Coliform groups showed high no. of colonies at 10^{-3} dilution, which decreased with increasing dilution.

Table 2 Microbial load of micro-organisms isolated from CD + CU+M+C

Organism	Dilution			
	3-Oct	10-4	5-Oct	6-Oct
Salmonella	-	-	-	-
E. Coli	-	-	-	-
Bacillus spp.	>300	57	6	3

CD: Cow Dung, **CU:** Cow Urine; **M:** Milk **C:** Curd

Difference of microbial growth from different components of PG on various media and effect of components of PG on microbial load

We next tested the growth of microbes in CD, CU, CD+CU, CD+CU+M+C and that of all components of PG together viz. CD+CU+M+C +G on different agars so that we could identify different microbial species based on growth on selection media. Thus if there were any clostridium species present, colonies would be present on clostridial medium and any gram-negative bacteria, such as pseudomonas would grow on cetrimide plates. As shown in Table 3, plates containing CD+CU mixture had most number of colonies present in almost all media except on cetrimide where CD gave rise to the most number of colonies. Addition of M+C decreased colony count on all media, whereas further addition of G i.e.

colonies derived from CD+CU+M+C+G increased the colony count only modestly.

Table 3 Difference in microbial growth from different components of PG on various growth media the effect of components of PG on microbial load.

Agar Type	MA	Cetrimide	SS	Clostridial	PDA
	No. of colonies x 10^2 observed				
CD	103	72	>300	> 300	74
CU	44	>300	98	120	154
CD + CU (1:2)	>300	0	>300	195	>300
CD + CU + M + C	80	0	137	36	48
CD + CU + M + C + G	98	16	170	128	198

Effect of sugars on microbial growth from the different components of PG

To further differentiate between the various bacterial species, we picked 3 colonies derived from CD, or CU, CD+CU 1:2 mixture, CD+CU+M+C and finally 6 colonies from all components of PG mixed together i.e the CD+CU+M+C+G mixture from various media plates and cultured them on media with different sugars and also conducted the indole test with them. Most of the colonies were capable of growth in all sugars, except for 1 colony which did not grow on glucose and mannitol and one colony which did not grown on maltose, as shown in Table 4.

Table 4 Effect of sugars on microbes from the different componenets of PG

Isolate no.	Source	Glucose	Sucrose	Mannitol	Lactose	Maltose	Indole test
1	CU	+	+	+	+	+	+
2	CU	+	+	+	+	+	+
3	CU	+	+	+	+	+	+
4	CD	+	+	+	+	+	-
5	CD	+	+	+	+	+	+
6	CD	+	+	+	+	+	+
7	CDCU	+	+	+	+	+	+
8	CDCU	+	+	+	+	+	+
9	CDCU	+	+	-	-	+	+
10	CDCUMC	+	+	+	+	+	-
11	CDCUMC	+	+	+	+	+	-
12	CDCUMCG	+	+	+	+	+	-
13	CDCUMCG	+	+	+	+	+	-
14	CDCUMCG	-	+	-	+	+	-
15	CDCUMCG	+	+	+	+	-	-
16	CDCUMCG	+	+	+	+	+	-
17	CDCUMCG	+	+	+	+	+	-

CD: Cow Dung, **CU:** Cor Urine; **CDCU:** Cow Dung + Cow urine **CUCDMC:** Cow Dung + Cow urine + Milk+ Curd, **CUCDMCG:** Cow Dung + Cow urine+ Milk +Curd + Ghee

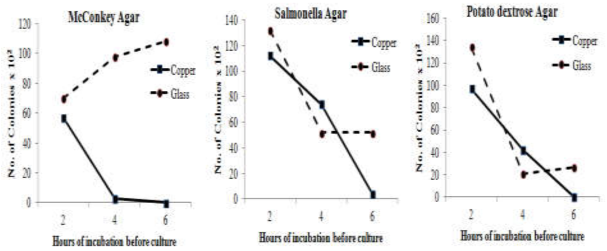

Fig. 2 Effect of using Copper vessel on the colony count from CD+ CU. CD+CU was prepared in the 2:1 ratio as described either in sterile glass or copper vessels and incubated at room temperature for 2, 4 or 6 hrs. 1 ml of the mixture from each vessel was plated out on corresponding agar plates. Plates were incubated for 48 hrs and colony count was noted.

Interestingly, colonies derived from the CD+CU+M+C mixture, irrespective of whether G was added or not, were all negative for the indole test.

Identification of the microbial species from various PG components or their mixtures

To identify the species of microbes isolated from the different PG components, we sent the same colonies isolated from different sources (as above, for Table 4) on slants for identification through sequencing their 16sRNA. 3 fungal colonies observed were also sent for identification. Table 5 shows the sequencing results from 3 colonies randomly chosen from a total of 20 samples that were sent. Table 6 shows the results of identification of the species identified from the 20 samples.

Effect of copper on PG

Since traditionally literature recommends fermentation of PG in copper vessels, we wanted to see the effect of mixing PG in a copper vessel instead of a glass vessel. We incubated the 1:2 CD+CU mixture, either in a sterile copper vessel or in a sterile glass beaker for 2 hrs, 4 hrs or 6 hrs and then cultured the microbes on different agars. As seen in Fig. 2, copper decreases the growth of microbes on MA, SS and PDA.

DISCUSSION

CD micro flora has been reported to contain abundant number of bacilli, lactobacilli and cocci and some identified and unidentified fungi and yeasts 2 We found a mixture of both gram positive and negative bacteria and rods and some bacilli species from CD, similar to that found by (Sharma & Singh, 2015 and Teo & Teoh, 2011). CD had the largest number of salmonella and gram positive clostridium species. Addition of CU to CD (CD+CU) decreased clostridial load from CD as seen by the colony count on clostridial agar. Clostridium possess typical characteristics of gram positive bacteria such as a thick cell wall. Cow urine has been found to possess lipase activity, which could be the key factor for it causing a reduction in the clostridium agar colony count from CD (Kumar *et al*. 2004).

CU had the largest number of gram negative growth on cetrimide plates. Surprisingly, addition of CD to CU (CD+CU) reduced the gram negative colony count from CU alone on cetrimide agar and plates containing diluents of CD+CU had the largest number of colonies in all agars except on cetrimide. (Table 3). CD has been reported to have anti-microbial activity, which could account for this observation.

Addition of M+C reduced the load in CD+CU, since colony count in CD+CU+M+C in all media was lower than that of CD+CU (Tables 1, 2 and 3). This was also observed when we conducted the indole test. All colonies derived from the combination of CD+CU+M+C were negative for the indole test. The bacetriocidal activity of curd has been well established and the mechanism is through lowering pH (Kotz *et al*., 1990). Milk fat has been reported to have anti-bacterial and anti-fungal activity (Subramanium, *et al*, 2005). Immunoglobulins, lactoferin, lysozyme, lactoperoxidase and vitamin B12 binding protein present in cow's milk possess antimicrobial effects.

Table 5 Sequencing of 16S RNA from microbial isolated from the different components of Panchagavya

Sr. No.	Sample Name	16 S Sequence obtained
1	CU-1	GCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGGAGATGAGGTGCTTGCACCTTATCTCAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTA GTGGGGGACAACATTCCGAAAGGAATGCTAATACCGCTTACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTGATAGATGAGCCTAAGTCGGATTAGCTAGTTGG TGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG GACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTGAGACTAATACTCTTGGATAGTGGA CGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGTGCGTAGGCGGCTTTTTAA GTCGGATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGGAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA
2	CU-2	CTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTG CCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGC TAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG AATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGATAAGGTTAATACCCTTGTT GATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTC TGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGA GATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGTGCGAAAGC
3	CU-3	TCTGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGC CTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATTATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGG CGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA GCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAA TAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGC GCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGA AATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAA
4	CD-1	Same as 2
5	CD-2	TGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGCTTGCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACT GCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAG CTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTAATACCTTTGC TCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT TTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAG AGATCTGGAGGAATACCGGTGGCGAAGGCG
6	CD-3	Same as 5
7	CDCU-1	TGAAAGACGGTTTCGGCTGTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGAT CGTAAAACTCTGTTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG GTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGACTTG AGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAA GC
8	CDCU-2	TGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTGGAAAGGAACGCTAATACCGCATACGTCCTACGGGCGAAAGTGGGGGATCT TCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAAC TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCAC TTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCG TTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTATGGCAG AGGG
9	CDCU-3	Similar to 5
10	CDCUMC-1	Similar to 5
11	CDCUMC-2	Similar to 3
12	CDCUMCG-1	Similar to 2
13	CDCUMCG-2	Similar to 2
14	CDCUMCG-3	TCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGG ATCTGCCCAGTCGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATGAACCCAGGTGGGA TTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG TGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGTAGCTAATAACT GCCAGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG CGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGC GTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGC
15	CDCUMCG-4	GCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTTGAGCGCTGAAGGTTGGTACTTGTACCGACTGGATGAGCAGCGAACGGGTGAGTAACGCGTGGGGAATCTGCC TTTGAGCGGGGGACAACATTTGGAAACGAATGCTAATACCGCATAAAAACTTTAAACACAAGTTTTAAGTTTGAAAGATGCAATTGCATCACTCAAAGATGATCCCGCGTTGTA TTAGCTAGTTGGTGAGGTAAAGGCTCACCAAGGCGATGATACATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCAG TAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACGTTGGTGAGAGTGGAAAG CTCATCAAGTGACGGTAACTACCCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAG GTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATGCATTGGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGC GTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAACTGACACTGAGGCTCGA
16	CDCUMCG-5	CTGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGGAGAGAGGGTGCTTGCACCTTAGCTCAGCGGCGGACGGGTGAGTAATGCTTAGGGAATCTGCCT ATTAGTGGGGGACAACATTCCGAAAGGGATGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGGATCTTCGGACCCTGCGCTCATAGATGAGCCTAAGTCGGATTAGCTAG TTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTTTGAGAGGATGATCCGCCACACTGGGAATGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT ATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCGCTCTAGGATAATACCCTAGATGCG TGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTACTGGGCGTAAAGCGCGCGTAGGTGGCTAA TTAAGTCAAATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA TCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCC
17	CDCUMCG-6	GGCGTGCCTAATACATGCAAGTCGAGCGAACGGACGAGAGTGCTTGCACTCTCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTATAAGACTGGGATAAC TTCGGGAAACCGGAGCTAATACCGGATAATATTTAGCTTCGCATGAAGCAATAGTGAAAGACGGTTCTGCTGTCACTTATAGATGGACCCGCGGTGTATTAGCTAGTTGGTGAG GTAACGGCTCACCAAGGCAACGATACATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACGTTAGTAACTGAACGTACCTTGACGGTA CCTTACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTCTCTTAAGTCTG ATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGA ACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAG
18	CDF-1	ACCGAGTGCGGGTCCTTTGGGCCCAACCTCCCATCCGTGTCTATTATACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTCGGCCGCCGGGGGGGCGCCTTTGCCCCCCGGGCCCG TGCCCGCCGGAGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAG AACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCAT TGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCA CATGCTCTGTAGGATTGGCCGGCGCCTGCCGACGTTTTCCAACCA
19	CUF-1	GCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATCGTACCTTGTTGCTTCGGCGGGCCCGCCTCACGGCCGCCGGGGGGCATCCGCCCCC GGGCCCGCGCCCGCCGAAGACACACAAACGAACTCTTGTCTGAAGATTGCAGTATGAGTACTTGACTAAATCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGGATCGA TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACACTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGC GTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCTCTCGCCCCCCGCTTCCGGGGGGCGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTT CGTCACCCGCTCTGTAGGCCCGGCCGGCGCCCGCCGGCGAACACCATCAAT
20	CDCUF-1	No sequence

Lu *et al*., 2014 identified organisms from the *Alcaligenes*, *Bacillus*, *Proteus*, *Pseudomonas Staphylococcus and Microbacterium* genera from 219 bacterial strains isolated from cow dung.

According to Ware *et al*. (1988), lower part of the gut of the cow contains various microorganisms including *Lactobacillus*, *Bacillus*, entero-cocci and yeasts. From the lot of microbes that we observed on the plates, we isolated and sequenced the enterococcus *Klebsiella*, and *E. coli* from CD (Table 5 & 6).

Table 6 Identification of organisms from sequencing of 16S RNA

Sr.No.	Sample Name	Sequence
1	CU-1	*Acinetobacter johnsonii*
2	CU-2	*Klebsiella pnuemoniae*
3	CU-3	*Bacillus licheniformis*
4	CD-1	*Klebsiella pnuemoniae*
5	CD-2	*Escherichia coli*
6	CD-3	*Escherichia coli*
7	CDCU-1	*Lysinibacillus xylanilyticus*
8	CDCU-2	*Pseudomonas stutzeri*
9	CDCU-3	*Escherichia coli*
10	CDCUMC-1	*Escherichia coli*
11	CDCUMC-2	*Bacillus licheniformis*
12	CDCUMCG-1	*Klebsiella pnuemoniae*
13	CDCUMCG-2	*Klebsiella pnuemoniae*
14	CDCUMCG-3	*Aeromonas veronii*
15	CDCUMCG-4	*Lactococcus lactis*
16	CDCUMCG-5	*Acinetobacter indicus*
17	CDCUMCG-6	*Macrococcus caseolyticus*
18	CDF-1	*Aspergillus tubinggensis*
19	CUF-1	*Penicillium oxalicum/Aspergillus fumigates*
20	CDCUF-1	----

CD: Cow Dung, **CU:** Cor Urine; **CDCU:** Cow Dung + Cow urine **CUCDMC:** Cow Dung + Cow urine + Milk+ Curd, **CUCDMCG:** Cow Dung + Cow urine+ Milk +Curd + Ghee, **CDF:** Fungal colony from dung, **CUF:** Fungal colony from urine**, CDCUF**: fungal colonies from combination of dung and urine.

Our sequencing results revealed the presence of *Acinetobacter, Klebsiella, Bacillus, Escherichia, Aeromonas, Lactococcus, Acinetobacter, Macrococcus, Aspergillus* and *Penicillium* genera from the colonies derived from CD, CU separately or a combination of CD+CU+M+C, similar to that reported by others.

Our study further revealed that when mixed together in a sterile Cu vessel as recommended by traditional literature, bacterial load came down further to virtually nil levels (Fig. 2).

Thus our study shows that traditional use of PG as a therapeutic agent against may be justified when executed under controlled conditions and as per the recommendations of traditional literature.

MA: MacConkey agar, **SS:** salmonella shigella agar, **PDA:** potato dextrose agar (PDA) or nutrient agar. **CD**: Cow Dung, **CU:** Cor Urine; **CDCU:** Cow Dung + Cow urine **CUCDMC:** Cow Dung + Cow urine + Milk+ Curd, **CUCDMCG:** Cow Dung + Cow urine+ Milk +Curd + Ghee. CD + CU 2:1 indicates that 4 gm of CD was mixed with 8 ml of CU, following which serial dilutions were conducted in sterile saline. All preparations were diluted serially to 10^{-2} in a sterile glass flask with sterile saline and incubated for 2 hrs at room temp before 1ml of the mixture was plated on corresponding media.

After culture of the organisms from CD or CU or their combinations, smears were prepared with saline on glass slides. Slides were gram stained and micrographed.

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