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Analysis of cow dung microbiota—A metagenomic approach

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Cow dung is being used from ancient times in agriculture as it has a significant role in plant growth promotion and plant protection. It is also being used in various religious practices as a purifier. Since only a small fraction of the total microbial diversity can be recovered by culturable methods, a culture independent 16S rDNA approach was taken up for more detailed analysis of cow dung microbiota. Total community DNA was extracted from fresh dung of Brown-Swiss breed and bacterial 16S rRNA genes were subsequently amplified, cloned, sequenced and deposited in GenBank. Bacteria belonging to the phyla Bacteroidetes (38.3%), Firmicutes (29.8%), Proteobacteria (21.3%) and Verrucomicrobia (2%) were identified. Bacteroidetes clones included the genera *Bacteroides*, *Alistipes* and *Paludibacter*; while *Clostridium*, *Ruminococcus*, *Anaerovorax* and *Bacillus* were predominant in Firmicutes. α- and γ-proteobacterial genera included *Acinetobacter*, *Pseudomonas*, *Rheinheimera*, *Stenotrophomonas* and *Rhodobacter*. The Verrucomicrobial clone showed high similarity to *Akkermansia*. Unculturable bacteria constituted 83.3% in the phylum Bacteroidetes and 87.5% in Firmicutes. All clones under phylum Proteobacteria were culturable bacteria. Eight per cent of the clone library represented previously uncharacterized and unidentified bacteria.

Keywords: Cow dung, metagenomics, phylogenetic analysis, 16S rRNA gene

Introduction

In India, cow dung is accepted as a purifier and has an important role in preserving environment. Besides being used as a fuel, it also finds use as a disinfectant in homes. Burning of cow dung is thought to repel mosquitoes. It also has a significant role in crop growth as manure because of humic compounds and fertilizing bioelements present in it[1]. The low C:N ratio in cow dung manure is an indication that it could be a good source of protein for the microbes involved in the decomposition of organic matter[2]. It is also a component of *Panchagavya*; it is a term used in Ayurveda to describe five important substances obtained from cow, namely, urine, dung, milk, ghee and curd. A number of formulations mentioned in Ayurveda describe the use of *Panchagavya* components either alone or in combination with drugs of herbal, animal or mineral origin[3]. Cow dung showed positive response in suppression of mycelial growth of plant pathogenic fungi like *Fusarium solani*, *F. oxysporum* and *Sclerotinia sclerotiorum*[4]. Cow dung extract spray was also reported to be effective for the control of bacterial blight disease of rice and was as effective as penicillin, paushamycin and streptomycin[5].

The primary reason for the lack of knowledge regarding the composition of the cow dung microbiome relates to the difficulty and expense of methods used to evaluate those populations[6]. Faecal bacteria in cattle have been analyzed using culture methods[7]. Culture based methods are extremely time consuming and to date we have only been able to culture approximately 1% of the bacteria present in animal gut[8]. Metagenomics is the culture-independent analysis of a mixture of microbial genomes (metagenome) using an approach based either on expression (functional analysis) or on sequencing (sequence-based analysis). Metagenomic analysis involves isolating DNA from an environmental sample, cloning the DNA into a suitable vector, transforming the clones into a host bacterium and screening the resulting transformants[9].

Here authors report the bacterial diversity and phylogenetic relationship of indigenous bacteria of cow dung by 16S rRNA gene libraries.

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Materials and Methods

DNA Extraction

DNA from fresh dung obtained from Brown-Swiss breed was extracted using the procedure of Proteus and Armstrong[10] with minor modifications. Cow dung (20 mg) was vortexed for 60 sec in 400 µL extraction buffer (200 mM Tris HCl-pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS), incubated at room temperature for 10 min, vortexed for 60 sec and centrifuged for 5 min at 12,000 rpm. The pellet was air dried and suspended in 50 µL water. Purified DNA was stored at −20°C for PCR amplification.

PCR Amplification and Cloning

PCR amplification was performed in 25 µL reaction volume, each containing 25 ng template DNA, 1× reaction buffer, 10 picomoles of primers, 0.4 mM dNTPs and 0.6 U of Taq DNA polymerase (Genei, Bangalore). The 16S rDNA primers used were 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387r (5′-GGG CGG WGT GTA CAA GGC-3′)[11]. Amplification conditions consisted of denaturation at 94°C for 90 sec, primer annealing at 55°C for 40 sec and primer extension at 72°C for 1 min. PCR was carried out for 30 cycles in Eppendorf PCR system and included an initial denaturation of 95°C for 3 min and a final elongation at 72°C for 20 min. Amplified 16S rDNA fragments were purified using PCR cleanup kit (Chromous Biotech, Bangalore) and were cloned in pGEM-T Easy vector (Promega WI, USA) according to manufacturer's protocol. Recombinant plasmids were transformed in *Escherichia coli* JM109 competent cells. White recombinant (47) colonies were selected at random and the presence of insert was confirmed using universal M13 primers[12].

DNA Sequencing and Phylogenetic analysis

Sequencing was carried out using an automated ABI 3100 Genetic Analyser at Genei, Bangalore. Universal sequencing primer T7 was used for 47 clones carrying the correct-sized insert (~1.5 kb). All sequences were compared with similar sequences of the reference organisms by BLAST search and chimeric sequences were removed on the basis of the results of the CHECK_CHIMERA program of the Ribosomal Database Project (RDP-II). Sequence data were aligned with the ClustalW package. Phylogenetic tree was constructed using the neighbour-joining method using Mega software.

Nucleotide Sequence Accession Numbers

The 16S rDNA sequences of 47 clones were deposited in Genbank under accession numbers HQ108059-HQ108091 and HQ144200-HQ144213 (Table 1).

Results and Discussion

A total of 47 16S rRNA gene clones were sequenced, representing the phyla Bacteriodetes, Firmicutes, Proteobacteria and Verrucomicrobia (Tables 1 & 2; Fig. 1). Clones were identified up to genus (29), family (5 clones), order (6), phylum (4) and none up to species level. Four clones were unculturable, unidentified bacteria.

Clones were predominantly Bacteriodetes (38.3%), a diverse and broadly distributed phylum including members present in both mammalian and insect guts, soil and both fresh and salt water ecosystems. A common feature associated with environmental bacteriodetes is their ability to degrade complex glycans, such as, cellulose, hemicellulose, chitin, agarose and alginate[13]. The genera *Bacteroides* (2%), *Alistipes* (15%) and *Paludibacter* (6%) were identified within this phylum. *Bacteroides* are well known intestinal bacteria that can be both beneficial and harmful. *Bacteroides* are also noted to participate in natural genetic transfer of antimicrobial resistance genes[6]. *Paludibacter* species consumes N-acetylglucosamine[14] and *A. putredinis* degrade fibre and glucosinolates[15].

Firmicutes (29.8%) were the second most abundant taxonomic group with clones grouping into the classes Clostridia and Bacilli. Within this Phylum the genera *Clostridium* (10%), *Ruminococcus* (2%), *Anaerovorax* (2%) and *Bacillus* (2%) were identified. *Clostridium* is a broad genus ubiquitous in the gastrointestinal tract. Clostridia can both positively and negatively influence the host animal. These effects are specifically associated with the individual *Clostridium* sp. involved. Many have negative influences on animal health including species like *C. perfringes, C. tetani, C. botulinum* and *C. difficile*[6]. Conversely, some *Clostridium* spp. may also be beneficial and improve digestion of complex organic materials, such as, cellulose[6], xylose[16], chitin[17] and lignocellulose[18], and even act as beneficial probiotics[6] and nitrogen fixers[19]. *R. flavefaciens* can degrade cellulose and xylan[20]. But some strains degrade mucin oligosaccharides in the human colon, thus decreasing host defense[21]. *Anaerovorax*, a strict anaerobe, is another genus obtained in this phylum. Earlier reports indicate that *Bacillus subtilis* obtained from fresh cow dung exhibited biocontrol activity against plant pathogenic fungi *F. oxysporum* and *Botryodioplodia theobromae*[22].

Table 1—Taxonomic distribution of 16S rRNA gene clones

Acc. no.	Organism	Phylum	Class	Order	Family	Genus	Closest cultured relative
HQ108069	Uncultured Bacteroidetes bacterium	Bacteroidetes	-	-	-	-	*Rikenellaceae* bacterium
HQ108061	Uncultured Bacteroidales bacterium	Bacteroidetes	Bacteroidia	Bacteroidales	-	-	-
HQ108062	Uncultured Bacteroidales bacterium	Bacteroidetes	Bacteroidia	Bacteroidales	-	-	-
HQ108071	Uncultured Bacteroidales bacterium	Bacteroidetes	Bacteroidia	Bacteroidales	-	-	-
HQ108085	Uncultured Bacteroidales bacterium	Bacteroidetes	Bacteroidia	Bacteroidales	-	-	-
HQ144205	Uncultured Bacteroidales bacterium	Bacteroidetes	Bacteroidia	Bacteroidales	-	-	-
HQ108077	Uncultured Rikenellaceae bacterium	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	-	-
HQ108074	*Bacteroides* sp.	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	*B. clarus*
HQ108059	Uncultured *Alistipes* sp.	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	*A. finegoldii*
HQ108078	Uncultured *Alistipes* sp.	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	*Alistipes* sp.
HQ108081	Uncultured *Alistipes* sp.	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	*A. putredinis*
HQ108086	Uncultured *Alistipes* sp.	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	*A. putredinis*
HQ108089	Uncultured *Alistipes* sp.	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	*A. massiliensis*
HQ144208	*Alistipes* sp.	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	*A. finegoldii*
HQ144210	*Alistipes* sp.	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	*A. massiliensis*
HQ108065	Uncultured *Paludibacter* sp.	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromoadaceae	*Paludibacter*	-
HQ108073	Uncultured *Paludibacter* sp.	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromoadaceae	*Paludibacter*	-
HQ108079	Uncultured *Paludibacter* sp	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromoadaceae	*Paludibacter*	-
HQ108088	Uncultured Firmicutes bacterium	Firmicutes	-	-	-	-	-
HQ144207	Uncultured Firmicutes bacterium	Firmicutes	-	-	-	-	-
HQ108080	Uncultured Clostridiales bacterium	Firmicutes	Clostridia	Clostridiales	-	-	*Clostridiales* bacterium
HQ108084	Uncultured Ruminococcaceae bacterium	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	-	-
HQ108087	Uncultured Ruminococcaceae bacterium	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	-	-

…Contd

Table 1—Taxonomic distribution of 16S rRNA gene clones...*Contd*

Acc. no.	Organism	Phylum	Class	Order	Family	Genus	Closest cultured relative
HQ108090	Uncultured Ruminococcaceae bacterium	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	-	-
HQ108070	*Clostridium* sp.	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*C. phytofermentans*
HQ108076	Uncultured *Clostridium* sp.	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*C. phytofermentans*
HQ108083	Uncultured *Clostridium* sp.	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*C. orbiscindens*
HQ144202	Uncultured *Clostridium* sp.	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*C. cylindrosporum*
HQ144213	Uncultured *Clostridium* sp.	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*Clostridium* sp.
HQ108082	Uncultured *Ruminococcus* sp	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*	-
HQ108075	Uncultured *Anaerovorax* sp.	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Anaerovorax*	-
HQ108091	*Bacillus* sp.	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*B. thuringiensis*
HQ108064	*Acinetobacter* sp.	Proteobacteria	γ-Proteobacteria	Pseudomonodales	Moraxallaceae	*Acinetobacter*	*A. baumannii*
HQ144211	*Acinetobacter* sp.	Proteobacteria	γ-Proteobacteria	Pseudomonodales	Moraxallaceae	*Acinetobacter*	*A. beijerncki*
HQ108063	*Pseudomonas* sp.	Proteobacteria	γ-Proteobacteria	Pseudomonodales	Pseudomonodacaea	*Pseudomonas*	*P. pseudoalcaligenes*
HQ108067	*Pseudomonas* sp.	Proteobacteria	γ-Proteobacteria	Pseudomonodales	Pseudomonodacaea	*Pseudomonas*	*P. putida*
HQ144203	*Pseudomonas* sp.	Proteobacteria	γ-Proteobacteria	Pseudomonodales	Pseudomonodacaea	*Pseudomonas*	*Pseudomonas* sp.
HQ144204	*Pseudomonas* sp.	Proteobacteria	γ-Proteobacteria	Pseudomonodales	Pseudomonodacaea	*Pseudomonas*	*Pseudomonas* sp.
HQ108066	*Rheinheimera* sp.	Proteobacteria	γ-Proteobacteria	Chromatiales	Chromataceae	*Rheinheimera*	*Rheinheimera* sp.
HQ108068	*Stenotrophomonas* sp.	Proteobacteria	γ-Proteobacteria	Xanthomonodales	Xanthomonodaceae	*Stenotropomonas*	*S. maltophilia*
HQ144206	*Rhodobacter* sp.	Proteobacteria	α-Proteobacteria	Rhodobacteriales	Rhodobacteriaceae	*Rhodobacter*	*Rhodobacter* sp.
HQ144209	*Rhodobacter* sp.	Proteobacteria	α-Proteobacteria	Rhodobacteriales	Rhodobacteriaceae	*Rhodobacter*	*Rhodobacter* sp.
HQ108072	Uncultured *Akkermansia* sp.	Verrucomicrobia	-	Verrucomicrobiales	Verrucomicrobiaceae	*Akkermansia*	*A. muciniphila*
HQ108060	Uncultured bacterium	-	-	-	-	-	-
HQ144200	Uncultured bacterium	-	-	-	-	-	-
HQ144201	Uncultured bacterium	-	-	-	-	-	-
HQ144212	Uncultured bacterium	-	-	-	-	-	-

Clones α- and γ-Proteobacteria represented 21.3% of the sequenced clones and the genera included *Acinetobacter* (4%), *Pseudomonas* (8%), *Rheinheimera* (2%), *Stenotrophomonas* (2%) and *Rhodobacter* (4%). *Acinetobacter* spp. are widespread in nature and some strains are known to be involved in biodegradation of a number of different pollutants and in the removal of phosphate or heavy metals. They are also well represented among fermentable bacteria for the production of economic products, such as, lipases, proteases, cyanophine, bioemulsifiers and several kinds of biopolymers. Furthermore, some strains were reported to produce IAA and siderophores. Several reports are available for phosphate solublisation and nitrogen fixation by *Acinetobacter*[18]. There are many reports on the production of antifungal compounds, IAA and siderophores by *Pseudomonas* spp.. *P. lini* could solublise phosphate and was used for

Table 2— Distribution of cow dung microbiome

Phylum	No. of clones (% of total)	Bacteria	No. of clones	
			Culturable	Non-culturable
Bacteroidetes	18 (38.3)	Phylum: Bacteroidetes	-	1
		Order: Bacteroidales	-	5
		Family: Rikenellaceae		1
		Bacteroides sp.	1	-
		Alistipes sp.	2	5
		Paludibacter sp.	-	3
Firmicutes	14 (29.8)	Phylum: Firmicutes	-	2
		Order: Clostridiales	-	1
		Family: Ruminococcaceae	-	3
		Ruminococcus sp.	-	1
		Clostridium sp.	1	4
		Anaerovorax sp.	-	1
		Bacillus sp.	1	
Proteobacteria	10 (21.3)	*Acinetobacter* sp.	2	
		Pseudomonas sp.	4	
		Rheinheimera sp.	1	
		Stenotrophomonas sp.	1	
		Rhodobacter sp.	2	
Verrucomicrobia	1 (2.1)	*Akkermansia* sp.	-	1
Unknown	4 (8.5)	Uncultured bacteria	-	4
Total	47		15	32

bioremediation of the hazardous pesticide, cypermethrin. However, they are potent human pathogens possibly causing respiratory, urinary and gastro-intestinal tract infections[23]. The biotechnological importance of *Stenotrophomonas* is partly due to its potential plant growth promoting effect like IAA production and consequent use in biological control of plant fungal diseases. Recent interest has also been focused on the capability of *Stenotrophomonas* to degrade xenobiotic compounds and its potential for decontaminating soil[24].

Within the phylum Verrucomicrobia, only one clone showing similarity to genus *Akkermansia,* an intestinal mucin degrading bacteria, was identified. Clone sequences identified as bacteria but otherwise unclassifiable represented 8% of the 16S rRNA gene library. These clones were very distinct from those of cultured organisms present in the NCBI database, which suggests high genetic diversity. Further studies are required to characterize and identify these organisms. Thus out of 47 clones sequenced, 32 (68%) showed homology with uncultured bacteria

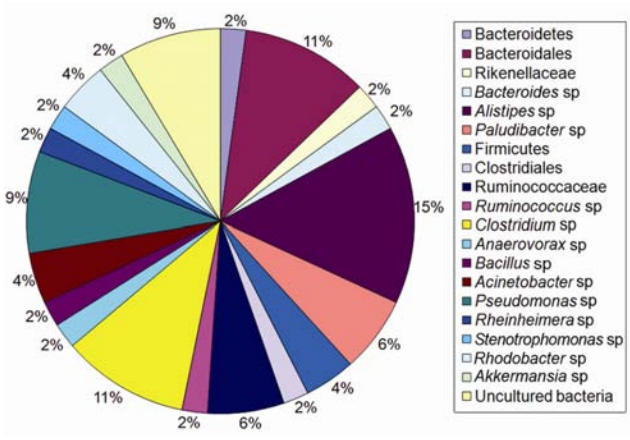

Fig. 1—Diagrammatic representation of cow dung microbiome.

and 15 (32%) with culturable bacteria. In phylum Bacteriodetes, 15 clones represented unculturable bacteria and 3 cultured ones. In Firmicutes, 12 clones showed homology to unculturable bacteria and two were cultured. All 10 clones in Proteobacteria were grouped as culturable bacteria and the only clone in Phylum Verrumicrobia as unculturable. Four cloned sequences (8%) did not show homology to any of the accessions in the NCBI database, indicating that these could be novel isolates (Table 1).

Although the similarity for most of the sequences with those of known bacteria was too low to identify the sequence as representing a particular species, a phylogenetic tree was constructed to investigate their taxonomic affiliation. The phylogenetic tree based on partial 16S rRNA gene placed 47 clones from cow dung sample into 3 groups–Firmicutes, Bacteroidetes and Proteobacteria (Fig. 2). Three clones, HQ 108060 (uncultured bacteria), HQ 108064 (*Acinetobacter* sp.) and HQ 144208 (*Alistipes* sp.) out-grouped. The Verrucomicrobial clone, *Akkermansia* sp. (HQ108072) also grouped under Firmicutes. Uncultured bacteria HQ144200 showed similarity to Firmicutes, while HQ144212 and HQ144201 to Bacteroidetes.

Bacterial diversity in faecal matter of cattle has earlier been assessed by two methods: culture-based and culture-independent metagenomic studies. The major difference between these two approaches is that culture-based experiments show bias to facultative anaerobes/aerobes, as they are easily cultivable under *in vitro* conditions. Strict anaerobes fail to grow in normal growth conditions and hence are often under-estimated in culture-based approach. The colony forming unit (cfu) counts of *E. coli* in faeces are typically in the 10^4 to 10^6 range, while total microbial

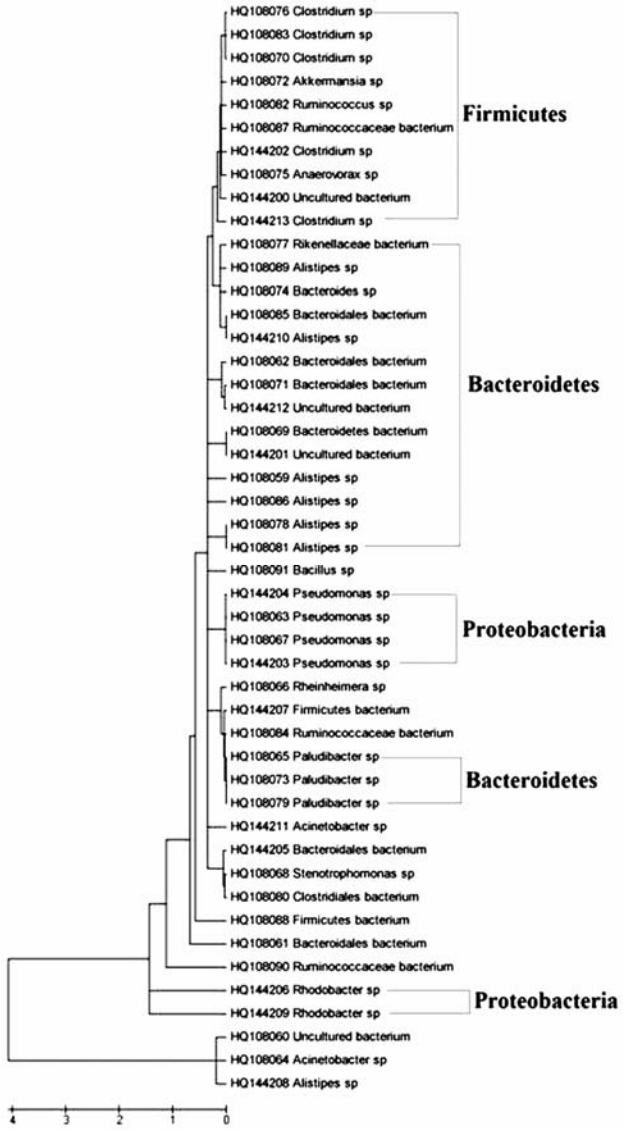

Fig. 2—Phylogenetic relationships of partial 16S rRNA gene sequences of clones recovered from cow dung sample.

counts are in the 10^{10} to 10^{11} bacteria/g of faeces range[6]. Culture-based methods that have been used in diversity studies estimate and over-represent the genera that can be grown easily *in vitro. E. coli* are easily cultured and ubiquitous in the faeces of animals so they are often used as a marker of faecal contamination in water supplies, however they typically comprise less than 1% of the intestinal bacterial population. Earlier metagenomic studies revealed that microbial population of lower intestine of cattle is dominated by strict anaerobes, such as, *Bacteroides, Clostridium* and *Bifidobacterium* sp., while facultative anaerobes, such as, the Enterobacteriaceae (*E. coli*), are typically 100-fold lower than the strict anaerobes[6].

In the present investigation, 31 clones (65%) out of 47 belonged to strictly anaerobic/anaerobic bacteria (*Alistipes*, *Paludibacter*, *Bacteroides*, *Clostridium*, *Ruminococcus*, *Anaerovorax* & *Akkermansia* sp.) and 14 clones (30%) belonged to aerobic/facultatively anaerobic bacteria (Table 2). Two clones were identified as *Rhodobacter* sp., an anaerobic photoautotroph/aerobic chemeoheterotroph. Earlier reports indicated that predominant species in bovine faecal matter included *Bacteroides*, *Clostridium*, *E. coli*, *Lactobacillus*, *Streptococcus* and Bifidobacteria[7]. But we failed to get *E. coli,* lactobacilli, streptococci and Bifidobacteria in the present study. These results are indicative of the culture-based bias inherent to studies enumerating the easily cultivable, facultatively anaerobic *E. coli in vitro*, while *Clostridium* and *Bacteroides* sp. are fastidious and typically require specialized anaerobic growth conditions.

Conclusion

An attempt was made to assess the diversity of microbes present in cow dung through a culture-independent, 16S rDNA sequencing approach. The predominant phyla detected in the study were Bacteroidetes, Firmicutes and Proteobacteria. Members of these phyla have been reported to be efficient degraders of complex organic mater like cellulose, lignin, chitin, xylan, etc. Hence, findings of the present investigation justify the use of cow dung in composting. This study also detected *Acinetobacter, Bacillus, Stenotrophomona* and *Pseudomonas* species, all of which have already been reported as IAA and siderophore producers. Many *Acinetobacter* and *Pseudomonas* species have been reported to have nitrogen fixing and phosphate solubilizing activities, thereby imparting plant growth promoting activity of cow dung, as observed by farmers. Several genera of bacteria identified in the study (*Bacillus* & *Pseudomonas*) are known for antagonistic properties against bacteria and fungi. This finding justifies the use of cow dung as a purifier in religious practices and for disease suppression in organic farming.

The metagenomic 16S rDNA library contained 68% clones representing unculturable bacteria that belonged to phyla Bacteroidetes, Firmicutes and Verrumicrobia. All clones within the phylum Preoteobacteria were culturable. Many strict anaerobes requiring specialized conditions to grow (*Clostridium, Bacteroides, Alistipes, Ruminococcus,*

Anaerovorax & Akkermansia) were also detected. Culture-based diversity analysis can detect only easily grown *E. coli,* Lactobacilli, Streptococci and Bifidobacteria, but may fail to detect fastidious and anaerobic bacteria. Hence, the study highlights the superiority of culture-independent metagenomic approach as a powerful tool for elucidating the diversity of animal microbiomes. Sequencing of more clones may give a clear and complete picture of cow dung microbiota. Furthermore, detailed studies are to be done to elucidate microorganisms which show no similarity to any identified culturable non-culturable microbe.

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