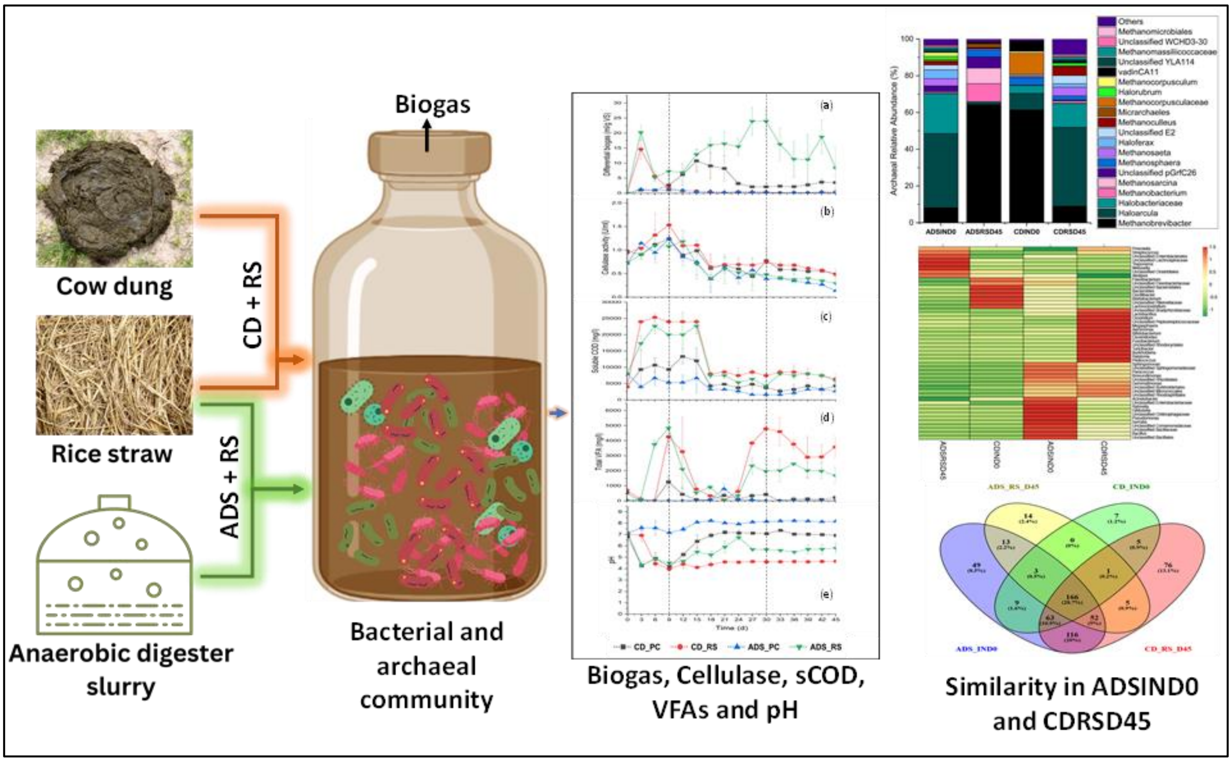
Chapter 4

Adaptability and diversity of core microbiome in evaluating the effect of digested versus raw manure in anaerobic digestion of rice straw



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4.1. Background

Anaerobic digestion (AD) of rice straw seems like a viable alternative to prevent its burning. It is a cost-effective and economically feasible method to produce biogas and biofertilizers from digestate for nutrient recycling [152] owing to its advances of reduced operating expenses, the ability to handle high levels of chemical oxygen demand (COD), minimal production of excess sludge, and the possibility of energy recovery [153]. Being a waste agricultural residue having low cost, renewability, easy availability, and biodegradability, RS is one of the most suitable feedstocks for AD to produce biomethane, the primary source of energy in biogas which can be used to produce electricity and fuel for cooking and vehicles [154]. However, the management and utilization of digestate have become challenging because of the steadily growing size and number of digesters [155]. The conversion of digestate to high-value products is another challenge because of the stark difference between manufacturing and selling costs [156]. Due to the presence of a microbial community that is enriched and acclimated and capable of reducing lag time and performing better than raw sludge and manures, certain portions of the digestate can be utilized as an inoculum source for the startup of smaller, local domestic and community digesters [157,158]. Rice straw generation is seasonal but abundantly available. It can be digested anaerobically at the farms in small digesters using the slurry from bigger digesters if the logistics and transportation problem is handled. This may help overcome the problem of pathogenic microorganisms and foul odour that arises because of open dumping of the digestate up to some extent.

The microbial community structure greatly influences the performance of an anaerobic digestion system [64]. Thus, the source and quality of inoculum play a vital role in governing the substrate degradation to fermentable sugars [65]. Cellulolytic enzymes called cellulases are responsible for the deconstruction of lignocellulose to reducing sugars like disaccharides and oligosaccharides, which are consumed by anaerobic microorganisms to produce methane through a series of complex reactions comprising *hydrolysis, acidogenesis, acetogenesis,* and *methanogenesis* carried out by bacteria and archaea [66]. Understanding the complex dynamics of microbial interactions in AD is very important to monitor the stability of the anaerobic digestion process. A suitable inoculum can enhance the degradation rate improve biogas generation and reduce the lag phase [67].

The rationale behind conducting this study was to establish synergism between microbial dynamics, biogas and methane production, enzymatic performance, sCOD, and volatile fatty acids (VFAs). Two different sources of inoculum containing active microbial communities, namely, raw cow dung (CD) and anaerobic digester slurry (ADS) from a local running digester using cow dung as a feedstock, were used for AD of rice straw. The hypothesis behind the current work is that process parameters combined with microbial dynamics can yield useful information on the bioconversion of lignocellulosic waste to bioenergy. The changes in morphology and composition of rice straw after anaerobic digestion were examined by Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). 16S rRNA metagenomic sequencing was used to study and compare the changes in the microbiome of two inoculum sources at the start and end of the anaerobic digestion of rice straw. The changes in the lignocellulosic components, namely, lignin, cellulose, hemicellulose, and silica content of RS, were also determined to estimate the extent of rice straw hydrolysis. This study may provide valuable insights into AD performance integrated with microbial dynamics and predict the stability of AD systems based on different process parameters.

4.2. Results and discussion

4.2.1. Biogas and methane production

Cumulative biogas production measured every 3 days from the anaerobic digestion of rice straw inoculated with raw CD and ADS is represented in *Figure 4.1(a)*, and significant differences were observed for 45 days. The highest cumulative biogas recorded was 197.13 ± 41.33 mL/g VS observed in ADS_RS contrary to 24.41 ± 3.94 mL/g VS in rice straw inoculated with cow dung (CD_RS) as inoculum (P-value $\leq$ 0.05). CD_PC produced the second-highest biogas volume of 137.56 ± 18.18 mL/g VS as compared to only 39.55 ± 3.53 mL/g VS in ADS_PC (P-value $\leq$ 0.01). *Figure 4.1(b)* shows the volume of biogas produced, recorded every three days. The peak biogas production values were observed on day 3 in CD_RS, ADS_PC, and ADS_RS, producing 416.33 ± 106.27, 58.67 ± 8.73, and 987.33 ± 46.11 mL biogas, respectively. Peak biogas production was observed on day 15 in CD_PC, giving 174.33 ± 19.86 mL biogas. The biogas production then decreased in all other samples except ADS_RS, where another peak was observed on day 27, giving 448.67 ± 161.51 mL biogas. The least cumulative biogas production was observed in CD_RS. The source of inoculum for anaerobic digestion affects biogas production, and it can be a differentiating factor when production at a commercial level is considered [76].

Figure 4.1(c) presents the cumulative methane production and *Figure 4.1(d)* shows the volume of methane produced measured every 3 days for a period of 45 days. ADS_RS produced maximum cumulative methane of 59.43 ± 14.75 mL/g VS which was 401.33 times higher than CD_RS, which produced negligible methane of 0.15 ± 0.02 mL/g VS (p-value $\leq$ 0.05). This indicates that the addition of rice straw collapsed the AD system as the methanogens were not acclimatized to utilize complex lignocellulosic biomass of rice straw validated by metagenomic sequencing data. CD_PC produced the second-highest methane volume of 36.69 ± 2.81 mL/g VS followed by 9.35 ± 1.37 mL/g VS in ADS_PC (P-value ≤ 0.01). The trend of volume of methane produced measured every three days resembled to that of biogas production, except that no production peaks for methane were observed in CD_RS. Even on day 3, when biogas production was as high as 416 mL, methane production was only 1.56 mL indicating the biogas mostly contained other gases. This clearly points towards the highest VFA accumulation resulting in a maximum pH drop at which methanogens couldn’t utilize the products of the acidogenic and acetogenic stages of AD. This also reduced the number of methanogens from day 0 to day 45. Non-methanogenic archaea like *Haloarcula*, *Halobacteriaceae*, etc., were dominant and the number of *Methanobrevibacter* reduced significantly in samples taken from CD_RS on day 45.

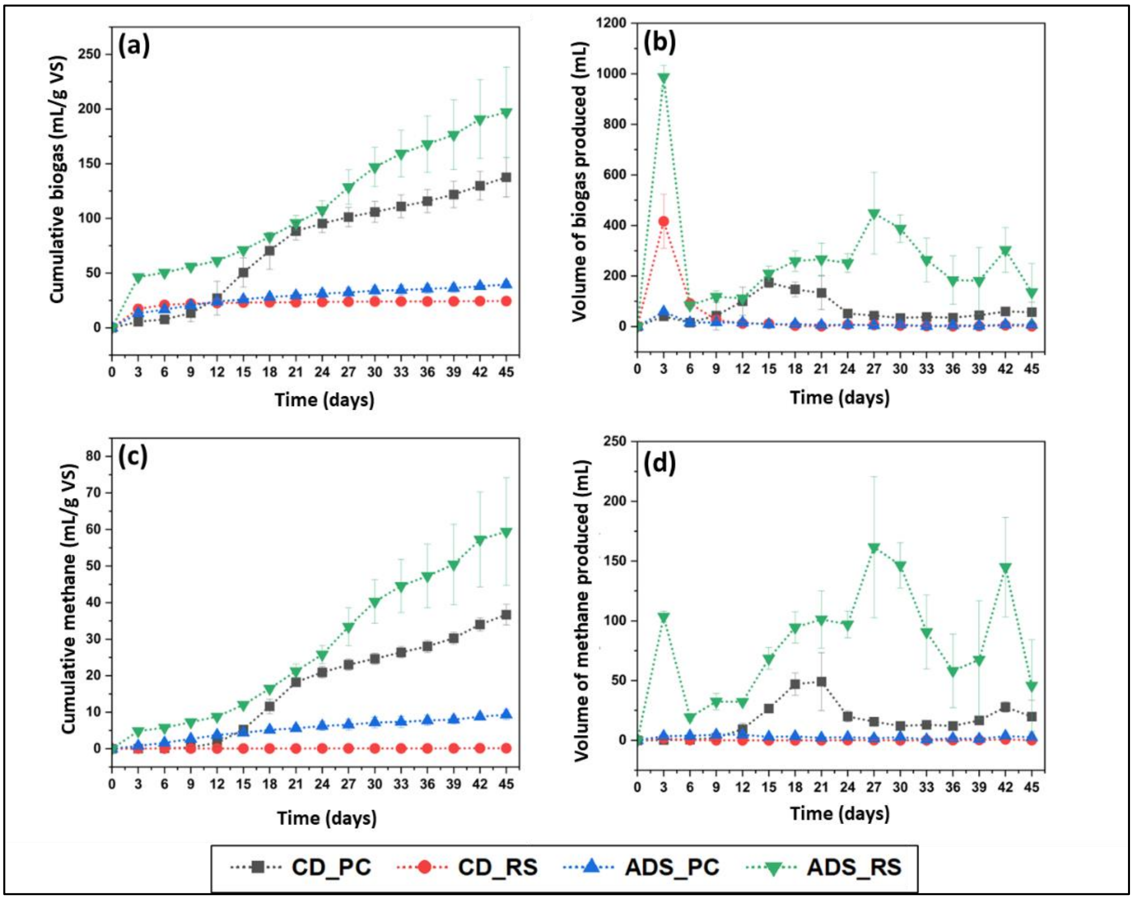

Figure 4.1. (a) Cumulative biogas, (b) volume of biogas produced (measured every three days), (c) cumulative methane, and (d) volume of methane produced (measured every three days) in anaerobic digestion of rice straw with cow dung (CD_RS) and anaerobic digester slurry (ADS_RS) as inoculum compared with controls CD_PC and ADS_PC

Figure 4.2(a) shows the trend of changes in cellulase activity for 45 days of anaerobic digestion of rice straw. Cellulases are extracellular enzymes that break down cellulose and hemicellulose present in lignocellulosic biomass into fermentable sugars in anaerobic digestion systems [159]. Higher cellulase activities were observed during the initial days of digestion which subsequently started decreasing. The maximum cellulase activity was observed on day 9 in CD_RS, CD_PC, and ADS_PC. However, ADS_RS had the highest cellulase activity on day 12. The highest cellulase activities were observed from day 3 to day 12 and then decreased as the days followed. These results indicate rapid hydrolysis rates during the initial days of anaerobic digestion. The cellulase activity in all the samples decreased as the days progressed, and CD_RS showed the highest activities contrary to the least biogas production values, which may result from increased production of VFAs, as shown in *Figure 4.2(c)*. High cellulase activities in CD_RS and ADS_RS were due to the presence of complex lignocellulosic rice straw in the AD system. The decrease in cellulase activities may result from the conversion of reducing sugars to volatile fatty acids (VFAs) in the anaerobic digestion system [143]. These enzymes are effective cellulose degraders and enable cells in the AD system to absorb hydrolysis products. The high abundance of *Clostridia* reported in metagenomic studies accounts for the cellulase activities as these are major players in the hydrolysis of cellulose [160].

Soluble chemical oxygen demand (sCOD) tells the concentration of soluble organics, which determines the efficiency of hydrolysis of the substrate to produce biogas. *Figure 4.2(b)* evaluates the change in sCOD concentrations during the anaerobic digestion of rice straw with two different inoculum sources. The daily concentration of sCOD showed a significant increase during the first 15 days indicating higher hydrolysis rates, and as the days progressed, the sCOD values dropped significantly. This can also be verified with the higher abundance of hydrolytic bacteria, including *Firmicutes, Proteobacteria, Bacteroidetes,* and *Spirochaetes,* in metagenomic sequencing results. The highest sCOD concentration of 25333.3 mg/L was observed on day 6 in CD_RS and 6666.66 mg/L on day 6 in ADS_RS. After that, the values showed a decreasing trend. The sCOD values showed lesser fluctuations after day 18. The trend in biogas production also showed a decrease with decreasing sCOD.

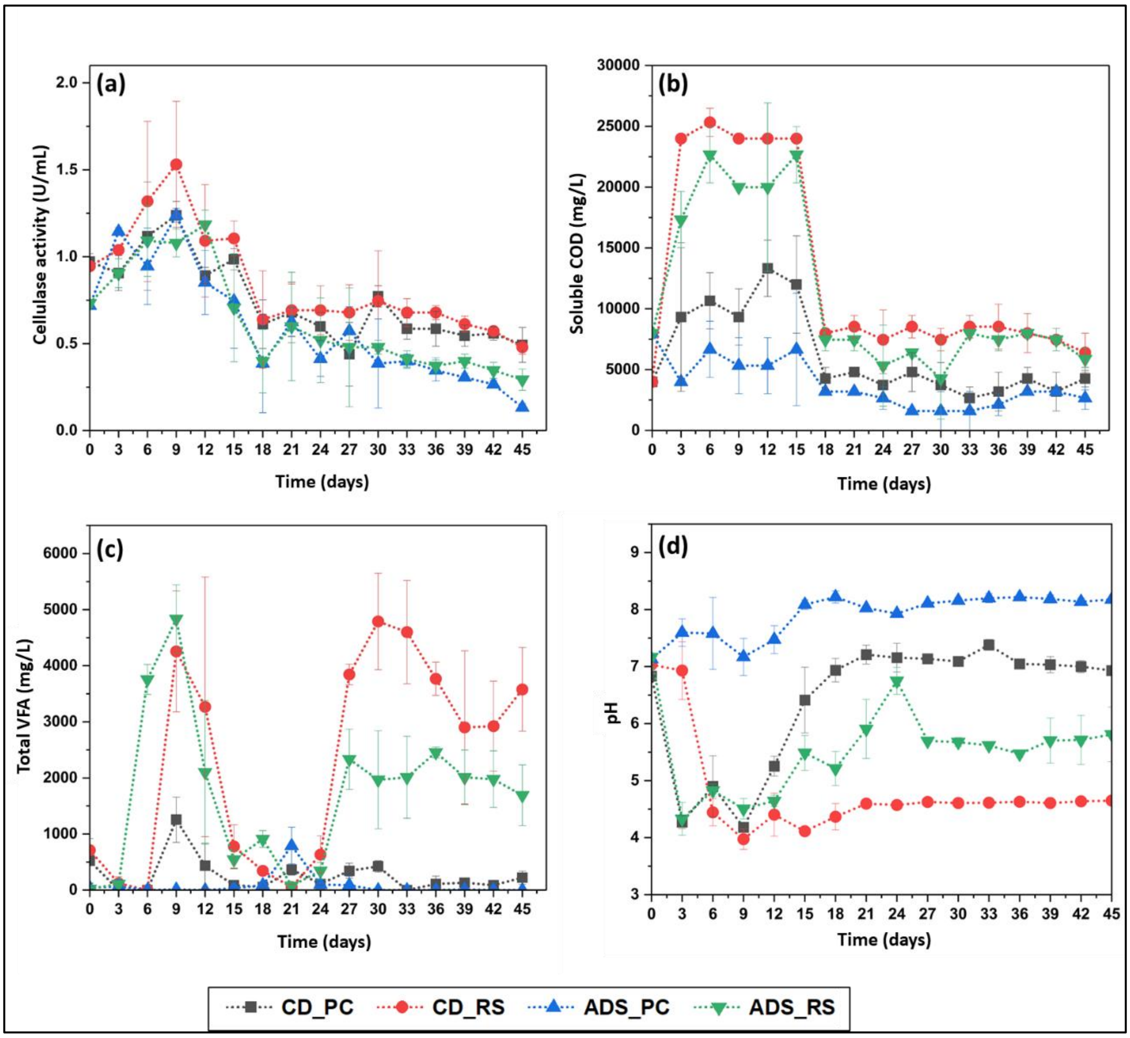

Figure 4.2. (a) Cellulase activity, (b) soluble chemical oxygen demand (sCOD), (c) volatile fatty acids (VFAs), and (d) pH in anaerobic digestion of rice straw with cow dung (CD_RS) and anaerobic digester slurry (ADS_RS) as inoculum compared with controls CD_PC and ADS_PC

Figure 4.2(c) shows the profile of VFAs produced in terms of acetic acid equivalents in the AD of rice straw with the two inoculum sources. The process of anaerobic digestion undergoes changes in VFA values, and it is one of the important parameters that directly impacts the functioning of the AD system. The range of VFAs in the system remained below 6000 mg/L, i.e., within the acceptable range to prevent inhibition [161]. Higher accumulation of VFAs in the AD process results in the reduction of pH, which may result in the failure of the AD process owing to the poor buffering of the system. Two peaks were observed during the AD process on day 9 and day 30. The VFA concentration first increased till day 9 and then started decreasing, and a second increase was observed post-day 24. VFA concentration was highest in ADS_RS (4833.33 ± 611.01 mg/L), followed by CD_RS (4255.56 ± 1077.72 mg/L) on day 9. During this phase, cellulase activity, and sCOD values were highest. The second highest peak was observed on day 30 in CD_RS (4788.89 ± 860.45 mg/L), followed by ADS_RS (2333.33 ± 536.45 mg/L) on day 27. However, the biogas production in CD_RS was lowest during this phase because it saw the maximum accumulation of VFAs and maximum drop in pH as shown in *Figure 4.2(d).* Methanogens thrive best in the pH range of 6.5 to 8, and at lower pH levels, methanogens fail to utilize the products of the acidogenic and acetogenic phases because of reduced enzymatic activity, accounting for the least production of biogas and the high sCOD values because of the lesser utilization of organic matter. ADS_RS, despite having high VFA values from day 0 to day 45, performed the best, which may indicate that AD community is better acclimatized and more resilient to reactor pH changes.

4.2.2. Changes in chemical and morphological characteristics of rice straw after digestion

There are a lot of cellulosic and hemicellulosic carbohydrates in rice straw that are easily converted into biogas. To evaluate the changes in the composition of rice straw, the cellulose, hemicellulose, lignin, and silica content of raw rice straw were compared with rice straw separated from digester bottles after 45 days of anaerobic digestion (*Table 4.1*). The cellulose content of RS increased by 1.67% in CD_RS_D45 and decreased by 2.67% in ADS_RS. Hemicellulose content increased in both samples. Increased values may indicate the sum of lignocellulosic components of RS combined with that of CD_IND0 and ADS_IND0. However, little reduction in lignin in ADS_RS and increment in CD_RS was observed. During the anaerobic digestion, the microbial community of ADS effectively broke down the lignocellulose in rice straw, reducing the lignin content in ADS_RS. On the other hand, increased lignin in CD_RS may be a result of the presence of undigested fibers of CD used as inoculum along with complex rice straw. Silica content was reduced by 2.5% and 0.8% in CD_RS and ADS_RS after 45 days of digestion, indicating the breakdown of silica. The decrease in lignocellulosic content indicates the dissolution of some of the lignocellulosic carbohydrates during the AD process. The reduction in degradation potential may result from the selectivity of microorganisms towards simpler carbohydrates present in the AD samples [93].

Table 4.1. Lignocellulosic components of raw rice straw and rice straw obtained after anaerobic digestion from samples having cow dung and anaerobic digester slurry as inoculum after 45 days of digestion

Sample	**Lignin (%)[a]**	**Cellulose (%)[a]**	**Hemicellulose (%)[a]**	**Silica (%)[b]**
Raw RS	8.53	43.30	36.36	45.60
CD_RS_D45	7.07	43.66	38.46	43.10
ADS_RS_D45	5.16	44.73	34.00	44.80

[a] Content of dry matter

[b] Content of ash

Images of SEM micrographs of (a, b) raw rice straw (RS), (c, d) rice straw digested with cow dung sampled on day 45 (CD_RS_D45), and (e, f) rice straw digested with anaerobic digester slurry sampled on day 45 (ADS_RS_D45) at a magnification of 3000 and 500, and a resolution of 10 µm and 50 µm respectively are shown in *Figure 4.3*. Usually, the surface of rice straw appears smooth with intact fibers on the surface but after digestion, it was observed that small holes on the surface of rice straw appeared [162], and broken fibers could be seen after 45 days of digestion, and these were more evident in the RS digested with ADS. Higher biogas production in ADS_RS may be a result of structure disintegration of RS. This may result from hydrolysis by microbial enzymes in the anaerobic inoculum that break down the lignocellulosic structure of rice straw. These results were similar to the observations reported in other studies [163]. The biogas volume in the samples containing RS and CD as inoculum was significantly less as compared to samples containing ADS as inoculum, and this is also evident in the SEM results as the exposure of the RS surface to microbial enzymes was less [93]. These results may also be validated by the sCOD values as the concentration of sCOD decreased as the anaerobic digestion progressed, indicating the consumption of decomposed lignocellulosic biomass that was the substrate for the anaerobic microbial community [164].

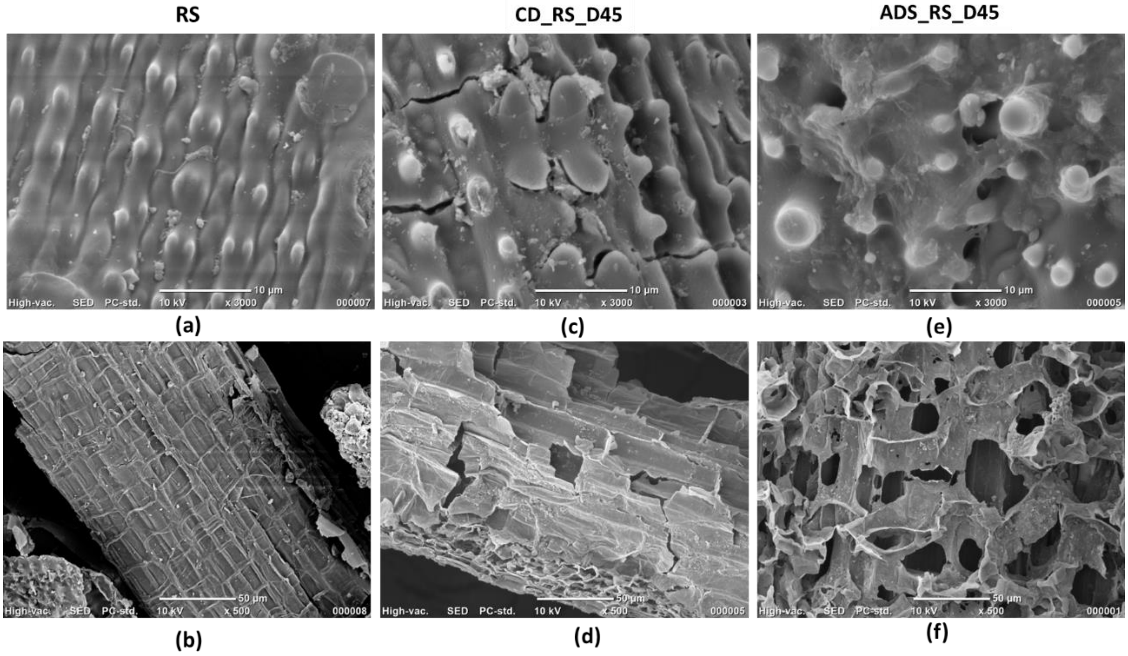

Figure 4.3. Images of SEM micrographs of (a, b) raw rice straw (RS), (c, d) rice straw digested with cow dung sampled on day 45 (CD_RS_D45), and (e, f) rice straw digested with anaerobic digester slurry sampled on day 45 (ADS_RS_D45) at a magnification of 3000 and 500, and a resolution of 10 μm and 50 μm

FTIR is generally used to study the changes in chemical bonds and functional groups. FTIR spectra of undigested rice straw (RS), rice straw digested with cow dung sampled on day 45 (CD_RS_D45), and rice straw digested with anaerobic digester slurry sampled on day 45 (ADS_RS_D45) were compared (*Figure 4.4*). The FTIR spectra for the three samples did not show considerable variations indicating that no significant changes in the chemical composition of rice straw occurred after digestion for 45 days. These results can also be validated with little changes in the lignin, cellulose, and hemicellulose concentration in RS, CD_RS_D45, and ADS_RS_D45 samples. There is a slight change in the intensity of the peaks at 2920 cm^{-1} for the three samples representing the changes in the C-H stretch of alkanes with methylene or methyl groups. The increase in absorption intensity in ADS_RS_D45 and CD_RS_D45 as compared to raw RS indicates that AD caused structural damage to the rice straw and exposed the cellulose content. Compared with the raw rice straw, the peak at 1732 cm^{-1} corresponding to the C=O stretch (maybe because of the release of hemicellulose) was more evident in the sample ADS_RS_D45 containing ADS as inoculum for rice straw hydrolysis, owing to the release of hemicellulose after digestion of rice straw. The increase in the absorption intensity of the peaks at 1514 cm^{-1} after digestion represents the presence of C=C stretching vibrations from the aromatic benzene rings of lignin. As cellulose and hemicellulose are broken down during AD, lignin-rich fractions remain, which must have resulted in increased aromatic peak intensity [165]. Sharp peaks at 1243 cm^{-1} refer to the cellulosic biomass in the samples indicating the availability of exposed cellulose after digestion [166].

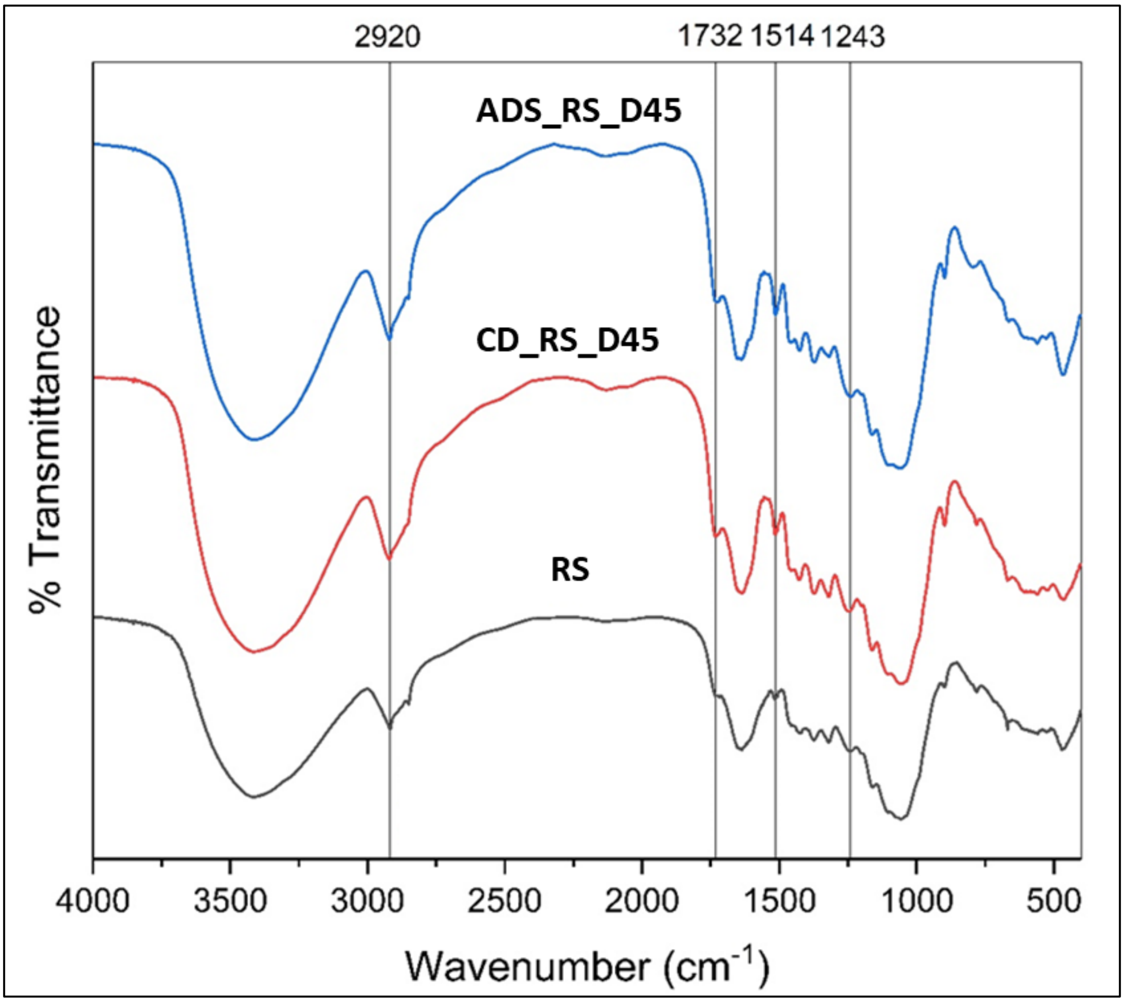

Figure 4.4. FTIR spectra of raw rice straw (RS), rice straw digested with cow dung sampled on day 45 (CD_RS_D45), and rice straw digested with anaerobic digester slurry sampled on day 45 (ADS_RS_D45)

4.2.3. Analysis of microbial community diversity

To study the changes in microbial community shift, 4 samples were taken for 16S rRNA genome sequencing, including ADS_IND0, CD_IND0, ADS_RS_D45, and CD_RS_D45. The microbial community in anaerobic digestion is very rich and diverse. The results of the microbial community before and after the anaerobic digestion of rice straw are presented in *Figure 4.5* and *Figure 4.6*.

4.2.3.1. Bacterial communities

According to the results of bacterial taxonomy at the phylum level described in *Figure 4.5(a)*, the dominant phyla were Firmicutes, Proteobacteria, Bacteroidetes, Spirochaetes, and Actinobacteria, which belong to the group of facultative anaerobes [167,168]. In the inoculum containing anaerobic digester slurry (ADS_IND0), the dominant phyla included 55.17% Proteobacteria, whose abundance decreased to 8.94% in ADS_RS_D45. Proteobacteria are associated with the carbon and nitrogen cycles in the natural environment and are reported to be associated with the increase in VFA production [169]. It was observed from Figure *4.2(c)* that VFA concentration was reduced in ADS_RS as compared to CD_RS as the days followed. The dominant phyla in the CD_IND0 were Firmicutes, and surprisingly, there was a negligible change in its abundance in the sample CD_RS_D45. Firmicutes share a syntrophic relationship with methanogens and are responsible for converting organic matter to VFAs and hydrogen. High accumulation of VFAs in CD_RS (*Figure 4.2(c)*) and negligible methane production (*Figure 4.1(d)*) indicate that Firmicutes maintained a stable population to produce VFAs, but the process did not proceed to the methanogenic stage. The relative abundance of Firmicutes increased from 21.57% in ADS_IND0 to 51.8% in ADS_RS_D45, accounting for the better degradation and higher biogas production as Firmicutes are reported to produce extracellular cellulase enzymes and play a crucial role in the metabolism of lignocellulosic biomass [170–172]. The increase in abundance of Bacteroidetes from 16.02% in ADS_IND0 to 20.15% in ADS_RS_D45 is also responsible for the increased biogas production as Bacteroidetes are reported to have the hydrolytic capability and can assimilate complex carbohydrates like cellulose and hemicellulose at neutral pH [173]. On the other hand, the relative abundance of Bacteroidetes decreased significantly from 39.16% in CD_IND0 to 14.55% in CD_RS_D45, explaining the reduced biogas production as a result of high VFA concentrations and acidification of the AD system creating stressful conditions for the microbial community This result explains the increased biogas production from rice straw in the sample containing anaerobic digester slurry over 45 days.

The relative bacterial abundance at the genus level (top 20) is described in *Figure 4.5(b)*. The most abundant microbial community genera belonged to the order of Unknown *Clostridiales,* followed by the genus *Prevotella.* The abundance of Unknown *Clostridiales* increased from 9.860% in ADS_IND0 to 38.39% in ADS_RS_D45 but decreased from 38.396% in CD_IND0 to 7.536% in CD_RS_D45. The potential reasons for the reduction in *Clostridiales* in CD_RS_D45 may be pH imbalance, excessive VFA accumulation and depletion of easily degradable carbohydrates. *Clostridioides* and *Clostridium* belonging to the order *Clostridiales* were significantly increased from day 0 to day 45, especially in CD_RS_D45. These belong to the phylum Firmicutes and play a crucial role in producing biogas owing to their high cellulolytic activity to produce volatile fatty acids from fermentable sugars [174]. They play a vital role in the production of hydrogen from fermented sugars. The abundance of *Prevotella* increased from 2.039% in ADS_IND0 to 14.47% in ADS_RS_D45, and the relative abundance was similar in the case of samples containing CD as inoculum. *Prevotella* is an efficient hydrolyzer of xylans, proteins, and peptides to produce organic acids, especially propionate [175]. The relative abundance of *Treponema* increased from 0.306% to 16.81% from day 0 to day 45 in samples containing ADS and decreased in samples containing the CD. *Treponema* belongs to the group of homoacetogens that feed on hydrogen (H_2) and carbon dioxide (CO_2) to convert to acetate [175]. The other dominant genera were *Serratia*, unknown *Bacteriodales*, *Enterobacteriaceae,* and *Streptococcus*.

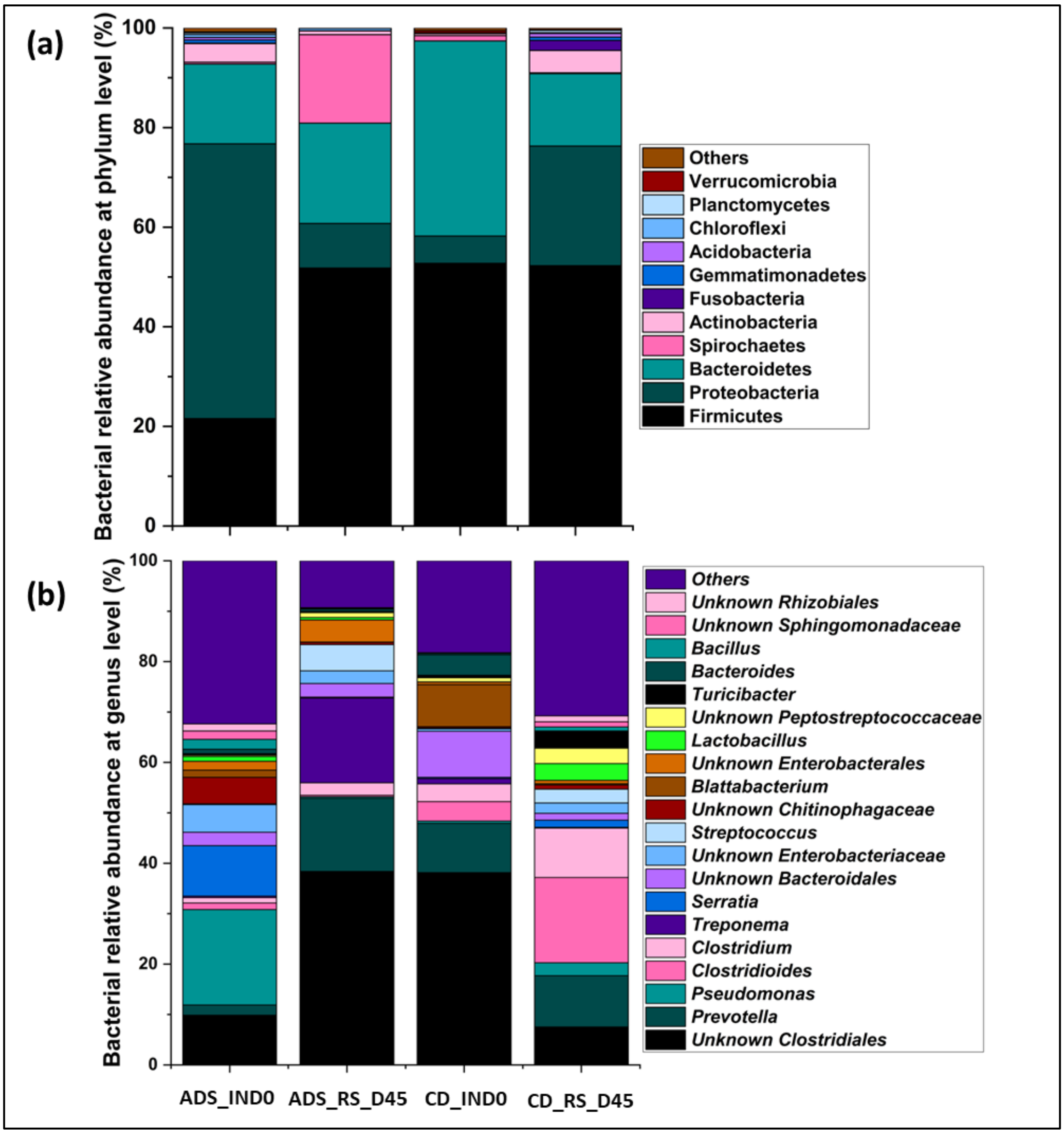

Figure 4.5. The analysis of the composition of bacterial community at (a) phylum and (b) genus level (top 20) before and after the anaerobic digestion of rice straw with CD and ADS as inoculum

4.2.3.2. Archaeal community

The changes in the archaeal community structure at the genus level are shown in *Figure 4.6*. The relative abundance of *Methanobrevibacter* showed a contrasting trend in the two inoculum sources. The abundance increased from 8.20% in ADS_IND0 to 64.257% in ADS_RS_D45, while the abundance decreased from 61.414% in CD_IND0 to 8.98% in

CD_RS_D45. *Methanobrevibacter* is strictly anaerobic hydrogenotrophic archaea responsible for producing methane from carbon dioxide and hydrogen. This indicates and validates the increased biogas production from rice straw over 45 days in samples containing anaerobic digester slurry as inoculum and a decrease in production in the samples containing raw cow dung as inoculum. The stark decrease from 40.33% in ADS_IND0 to 1.025% in ADS_RS_D45 in the abundance of *Haloarcula*, a halophilic archaeon promoted anaerobic digestion as *Haloarcula* are usually aerobic but can grow in anaerobic conditions as well. On the other hand, there was an increase in the abundance of *Haloarcula* from 9% in CD_IND0 to 43% in CD_RS_D45, explaining the decrease in the production of biogas. They can convert sugars to organic acids during the acidogenic phase [175].

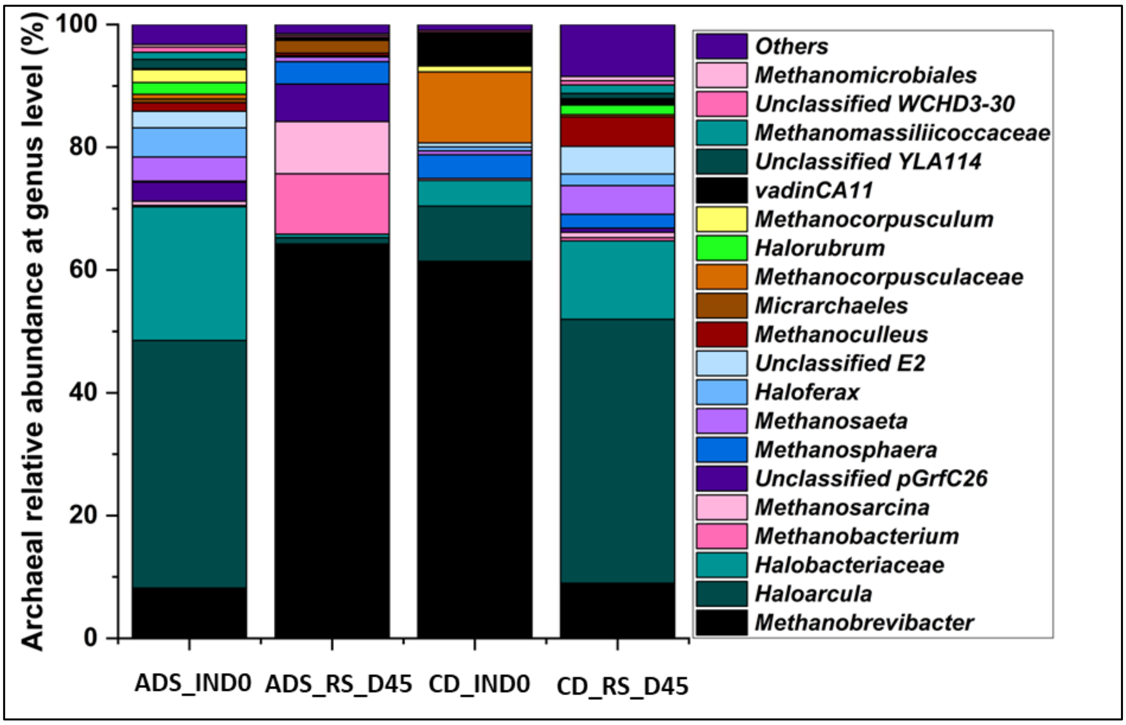

Figure 4.6. The analysis of the composition of archaeal community at the genus level (top 20) before and after the anaerobic digestion of rice straw with CD and ADS as inoculum

An increase in abundance of the archaeal genus *Methanobacterium* from 0.25% in

ADS_IND0 to 9.84% in ADS_RS_D45 was observed. Their abundance in CD_IND0 and CD_RS_D45 was almost similar. The low abundance of *Methanosaeta* as compared to *Methanobrevibater* might be responsible for the hydrogenotrophic production of methane and lesser production from the acetoclastic pathway. The abundance of *Methanosarcina,* a versatile anaerobic methanogen that can produce methane from all three methanogenic pathways, also increased from 0.67% in ADS_IND0 to 8.51% in ADS_RS_D45. However, there was no significant increase in samples containing CD as inoculum. *Methanosphaera, Methanoculleus,* and some unclassified methanogenic archaea were also detected. To summarise, changes in the microbial community at the start and end of the anaerobic digestion experiment with two different inoculum sources were evident, and one very interesting observation between the archaeal community structure of ADS_IND0 and CD_RS_D45 was the similarity between the abundance of *Methanobrevibacter, Haloarcula, Methanobacterium,* and *Methanosaeta* indicating the advantage of using digested cattle manure for anaerobic digestion. The handling and managing of slurry from the continuously running digesters is a major challenge in the success of anaerobic digestion for energy generation because of poor supply versus demand for digestate. The digestate from bigger digesters can serve as a feeder for the smaller domestic and community digesters.

4.2.3.3. Comparative analysis of microbial community diversity

The correlation between the different bacterial genera (top 50) present in the 4 samples is represented using the heatmap in *Figure 4.7*. The heatmap shows a strong positive correlation between the bacterial genera of ADS_IND0 and CD_RS_D45 showing some common bacterial members like *Sphingomonas, Paracoccus, Brevundimonas,* Unclassified *Rhizobiales, Burkholderia, Turicibacter* etc., while others had less correlation. The core microbiome of the AD samples is given by the heatmap in *Figure*

4.8, showing the highest abundance of Unclassified *Clostridiales*, *Prevotella*, *Clostridium*, and Unclassified *Bacteroidales,* followed by *Clostridioides* and Unclassified *Enterobacteriaceae. Prevotella* has been linked to using plant cell-wall material to hydrolyse lignocellulosic components like xylans and protein metabolism [176].

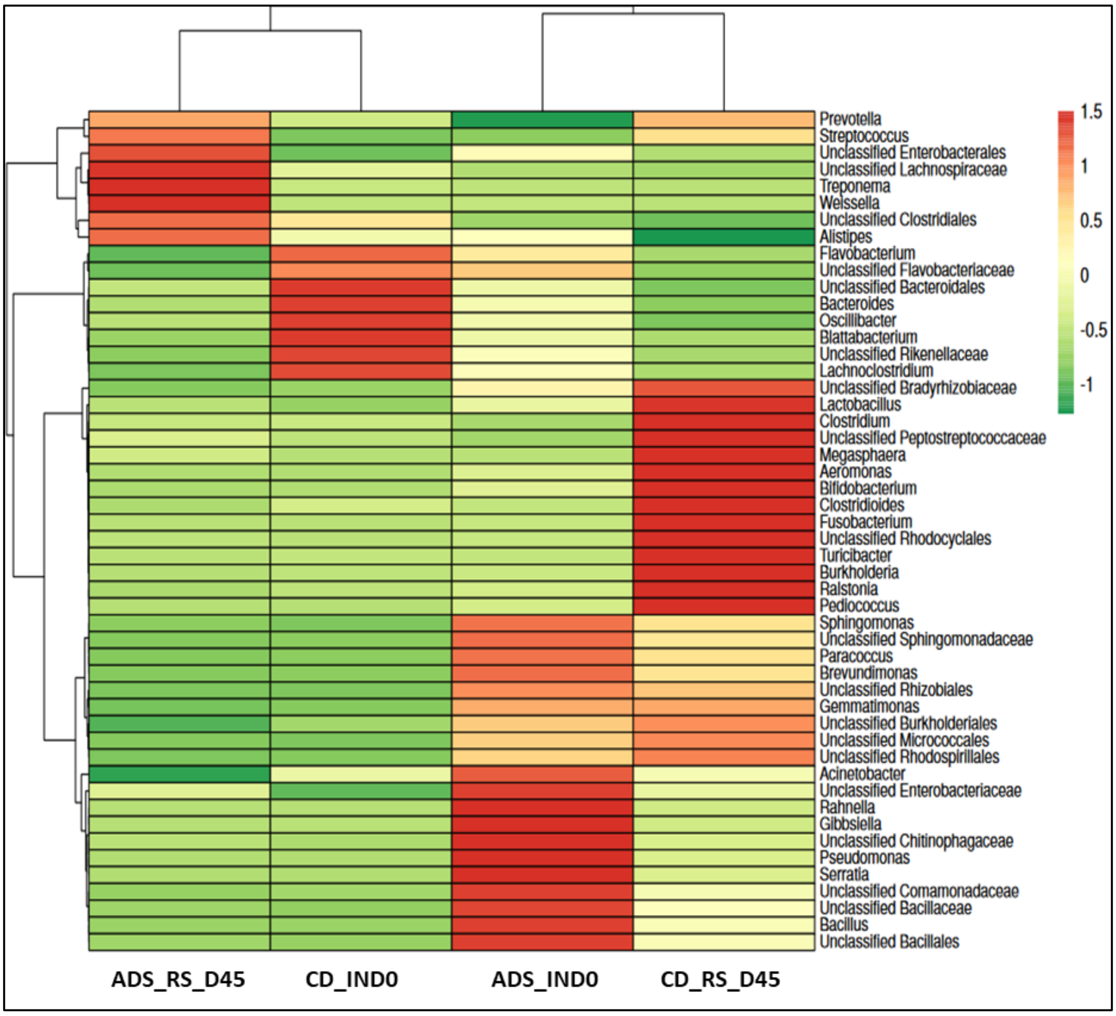

Figure 4.7. Correlation heat map of the top 50 genera in the anaerobic digestion of rice straw with cow dung (CD) and anaerobic digester slurry (ADS) as inoculum compared with controls CD_PC and ADS_PC

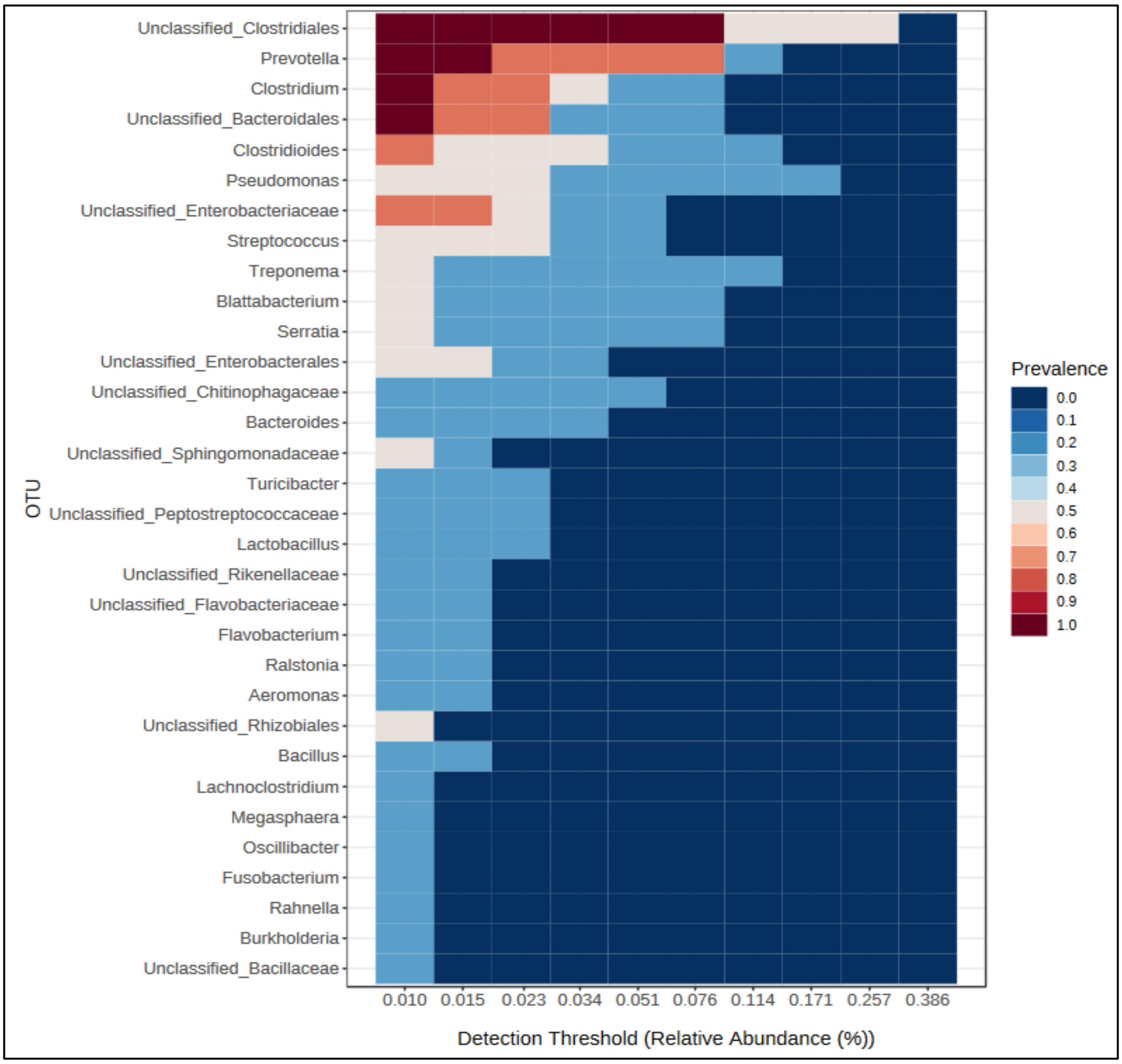

Figure 4.8. Heatmap of the core microbiome in the anaerobic digestion of rice straw with cow dung (CD) and anaerobic digester slurry (ADS) as inoculum compared with controls CD_PC and ADS_PC

The shared bacterial operational taxonomic units (OTUs) in the 4 different samples are presented using a Venn diagram shown in *Figure 4.9*. The numbers presented represent the number of similar OTUs and their percentage between different groups. 116 common bacterial entities were detected among ADS_IND0 and CD_RS_D45. On the other hand, ADS_IND0 and CD_IND0 show just 9, and ADS_RS_D45 and CD_RS_D45 show just 5 common bacterial genera, indicating the least correlated microbial community. All 4 groups shared 166 OTUs. Distinct OTUs in ADS_IND0 were 49, 14 in ADS_RS_D45, 7 in CD_IND0, and a maximum value of 76 in CD_RS_D45. This explains the dynamic behavior of the microbial consortia that keeps changing with time under different operating conditions. The changes in the microbial community are more rapid at the start of the anaerobic digestion process explaining higher fluctuations in the biogas, cellulase, and sCOD values, and become stable as the process continues with each passing day representing a stable community [177]. Rarefaction curves represent the richness of species in different samples. It can be seen that the species richness increased in CD_RS_D45 as compared to CD_IND0 and decreased in ADS_RS_D45 as compared to ADSIND0.

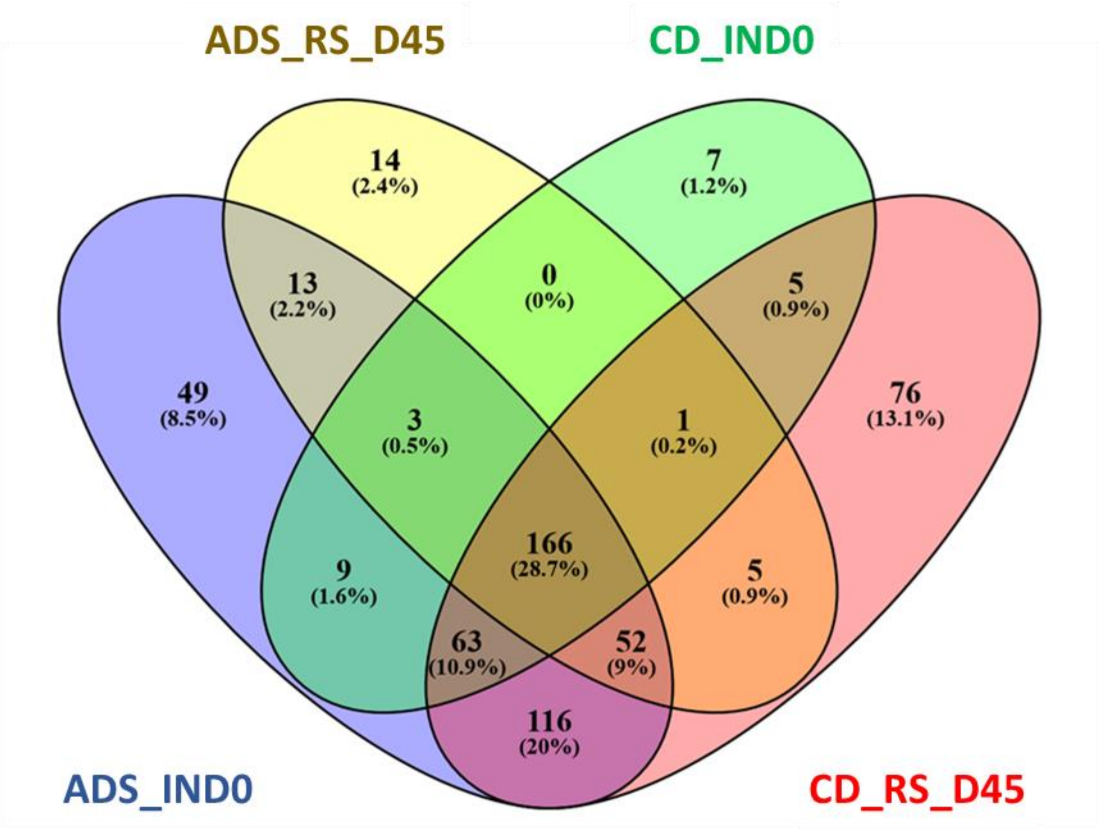

Figure 4.9. Venn diagram for the distribution of shared bacterial OTUs among the four experimental samples

The diversity and richness of microbial community in a sample were evaluated based on microbial composition using four different indices, namely, Chao 1, Shannon, Simpson, and Fisher index, and are represented in *Table 4.2*. The change in diversity of the microbial community is also represented using rarefaction curves (*Figure 4.10*). Chao 1 is a non-parametric estimator that uses the frequency of rare species. Fisher's alpha is a parametric estimator that utilizes a probability distribution to model the abundance of species. The Shannon index employs both species richness and evenness, while the Simpson index calculates the likelihood, ranging from 0 to 1, that two randomly chosen individuals from the same sample belong to the same species. The higher the value of the alpha indices, the higher the diversity of the community [178]. The results show that the value of alpha diversity indices decreased in ADS_RS_D45 as compared to ADS_IND0, indicating that some of the bacteria were eliminated and others enriched during the digestion period because of natural selection. A reverse trend was observed in samples containing cow dung as the alpha diversity increased from CD_IND0 to CD_RS_D45, indicating the enrichment and evolution of the microbial communities.

Table 4.2. The distribution of alpha diversity indexes of microbial communities

Sample	**Chao 1**	**Shannon**	**Simpson**	**Fisher**
ADS_IND0	353.600	3.803	0.934	50.323
ADS_RS_D45	213.857	2.272	0.794	24.415
CD_IND0	246.100	2.639	0.827	29.009
CD_RS_D45	333.000	3.701	0.938	46.783

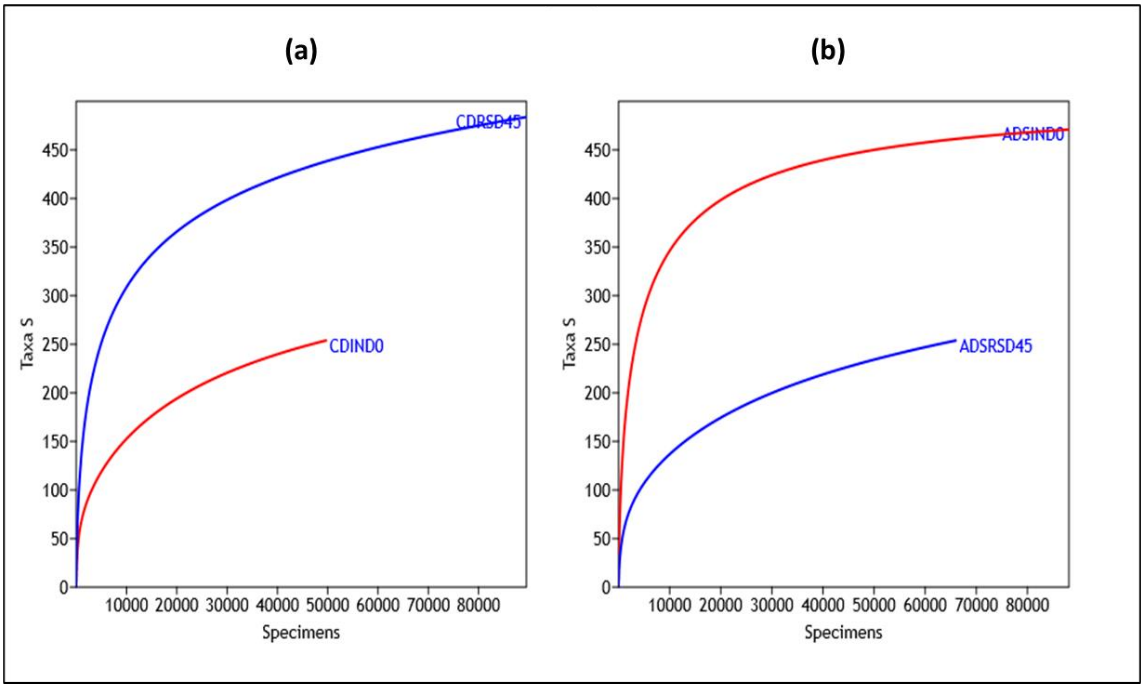

Figure 4.10. Rarefaction curves for samples (a) ADS_IND0, and ADS_RS_D45, and (b) CD_IND0, and CD_RS_D45

4.3. Conclusion

Most studies that evaluate how different types, age, and acclimatization of inoculum affect the digestion of lignocellulosic biomass do not account for the diversity and adaptation of the microbiome. However, this study compares the digestive performance of raw versus digested manure and offers strong evidence that highlights the outperformance and higher process stability of acclimatized inoculum in determining the rate of feedstock degradation, which is essential for achieving higher energy recovery from lignocellulosic residues. It was observed that the relative abundance of dominant microbial genera after 45 days of digestion of rice straw inoculated with raw CD was similar to the ADS inoculum used at the start of the experiment indicating inoculum acclimatization and aging plays a crucial role in AD. In our study, rice straw digested with ADS produced 7.07 times higher biogas (197.13 ± 41.33 mL/g VS) and showed higher sCOD utilization. Samples that used raw CD as inoculum for AD of rice straw showed the highest accumulation of volatile fatty acids (VFAs), compromising the buffering capacity of the AD system resulting in the least biogas production (24.41 ± 3.94 mL/g VS) as compared to samples containing ADS as inoculum. Metagenomic studies indicated that a higher abundance of hydrolyzers and hydrogenotrophic methanogens in ADS resulted in better performance of AD of rice straw. Implementation of the knowledge contributed from this study may not just help with the management and utilization of the digestate but also enhance energy recovery from crop residues.