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Molecular insights informing factors affecting low temperature anaerobic applications: Diversity, collated core microbiomes and complexity stability relationships in LCFA-fed systems

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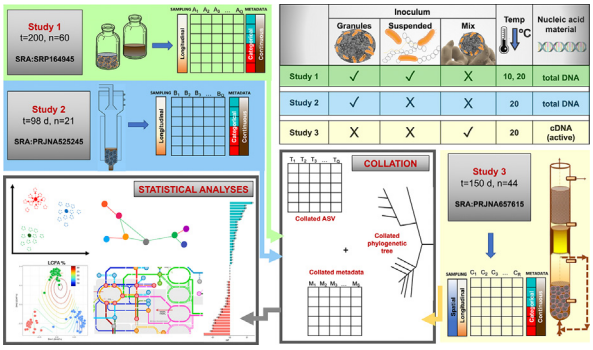
HIGHLIGHTS

- Comparative analysis performed for anaerobic low temperature long chain fatty acids degrading microbiomes
- Variation in the richness and diversity of the microbiomes linked to inoculum characteristics
- Core archaeal genera included 3–7 taxa, including *Methanobacteria* and *Methanosaeta*.
- Core bacterial taxa are complex (>2000), fermentative, many with unknown functions.

GRAPHICAL ABSTRACT



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ABSTRACT

Fats, oil and grease, and their hydrolyzed counterparts-long chain fatty acids (LCFA) make up a large fraction of numerous wastewaters and are challenging to degrade anaerobically, more so, in low temperature anaerobic digestion (LtAD) systems. Herein, we perform a comparative analysis of publicly available Illumina 16S rRNA datasets generated from LCFA-degrading anaerobic microbiomes at low temperatures (10 and 20 °C) to comprehend the factors affecting microbial community dynamics. The various factors considered were the inoculum, substrate and operational characteristics, the reactor operation mode and reactor configuration, and the type of nucleic acid sequenced. We found that LCFA-degrading anaerobic microbiomes were differentiated primarily by inoculum characteristics (inoculum source and morphology) in comparison to the other factors tested. Inoculum characteristics prominently shaped the species richness, species evenness and beta-diversity patterns in the microbiomes even after long term operation of continuous reactors up to 150 days, implying the choice of inoculum needs careful consideration. The generalised additive models represented through beta diversity contour plots revealed that psychrophilic bacteria RBG-13-54-9 from family *Anaerolineae*, and taxa WCHB1–41 and *Williamwhitmania* were highly abundant in LCFA-fed microbial niches, suggesting their role in anaerobic treatment of LCFAs at low temperatures of 10–20 °C. Overall, we showed that the following bacterial genera: *uncultured Propionibacteriaceae*, *Longilinea*, *Christensenellaceae R7 group*, *Lactivibrio*, *candidatus Caldatribacterium, Aminicenantales*, *Syntrophus*, *Syntrophomonas*, *Smithella*, RBG-13-54-9, WCHB1–41, *Trichococcus*,

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Proteiniclasticum, SBR1031, *Lutibacter* and *Lentimicrobium* have prominent roles in LtAD of LCFA-rich wastewaters at 10–20 °C. This study provides molecular insights of anaerobic LCFA degradation under low temperatures from collated datasets and will aid in improving LtAD systems for treating LCFA-rich wastewaters.

1. Introduction

Fats, oils and grease (FOG) constitute a major organic fraction of various wastes and wastewaters generated from food preparation (restaurant, cafeteria) and processing, oils processing (palm oil, olive oil etc.), dairy, slaughterhouse and wool scouring (Holohan et al., 2022). Certain wastewaters, typically considered as carbohydrate-rich, for example, corn to ethanol thin stillage and municipal wastewaters may also constitute high concentrations of FOG (Dereli et al., 2015; Keating et al., 2018; Petropoulos et al., 2018). Typical estimates of municipal wastewaters generated per capita are 120–170 L per day, containing 50–150 mg/L of FOG (Holohan et al., 2020; Petropoulos et al., 2018). Another example of a high wastewater generating industry is dairy processing, wherein conservative estimates suggest generation of about 0.4-trillion liters of dairy wastewaters annually with the FOG fraction ranging from 0.3 to 40 g per liter of wastewater (Singh, 2019). This FOG fraction is an important substrate for anaerobic digestion due to its higher methane production potential relative to the carbohydrate and protein-fractions (Alves et al., 2009). Furthermore, majority (~90 %) of the total organic carbon, and thus, the methanogenic potential of FOG is conserved within the hydrolysis products - long chain fatty acids (LCFAs) (Hanaki et al., 1981), which indicates LCFAs as key substrates in the anaerobic FOG degradation pathway. However, a number of challenges are associated with the anaerobic digestion of LCFAs including: i) sludge flotation and washout induced by LCFAs; ii) mass-transfer limitations imposed by formation of hydrophobic LCFA layer around sludge aggregates (Singh, 2019); iii) decrease in cell permeability (Zhou et al., 2013); iv) inhibition of enzyme activity (Zheng et al., 2005); v) disruption of cellular energy functions; vi) increased solubilization of microbial lipid bilayer and membrane proteins (Desbois and Smith, 2010). Overall, the impact is reduced methane production and treatment efficiency due to the inhibition of multiple trophic groups in the anaerobic digestion pathway- hydrolytic bacteria, syntrophic bacteria and methanogenic archaea (Davidsson et al., 2008; Hwu and Lettinga, 1997; Lalman and Bagley, 2001, 2000; Sun et al., 2013).

High-rate low temperature anaerobic digestion (LtAD) of wastewaters at their discharge temperatures has the potential to achieve energy neutrality (Gao et al., 2014; McKeown et al., 2009). However, at temperatures ≤20 °C, the challenges associated with LCFA degradation are exacerbated due to a decrease in LCFA solubility, substrate uptake kinetics and mass transfer, and impeded mixing due to the increase in bulk viscosity (Singh, 2019). Maintaining growth rates and activities of syntrophic bacteria and methanogens is fundamental to LtAD operation and process stability, however these microbes have inherently slow growth rates which further decrease at low temperatures (Cai et al., 2021). Previously, the abundance and activity of key microbial taxa has been linked to improved functionality of LtAD processes by application of various strategies such as microbial acclimation, optimization of operational parameters or utilization of specialized reactor designs (Bialek et al., 2014; Dev et al., 2019; Park et al., 2012; Wang et al., 2020). Acclimation of inoculum to treatment environment, such as substrate and operational characteristics, has been shown as a useful strategy in enhancing the LtAD applications (Darko et al., 2022; Kurade et al., 2020; Lendormi et al., 2022). Based on the understanding that the type of inoculum seeded at start up may affect the functional potential and adaptability of the anaerobic microbiome during reactor operation, inoculum sourced from psychrophilic conditions (~5 °C) have been used for LtAD of various substrates. Inoculum obtained from frozen natural sites were employed for seeding anaerobic reactors treating brewery and municipal wastewaters at 15 °C (Petropoulos et al., 2019; Xing et al., 2010). Alternatively, inoculum pre-adapted to psychrophilic conditions were used, for example, granular sludges acclimated to 17–20 °C were used for treating volatile fatty acids (VFA)-based and carbohydrate-rich wastewaters in expanded granular sludge bed reactors (EGSBs) at 5–15 °C (Esparza-Soto et al., 2013; Syutsubo et al., 2008).

For any AD system, inoculum and substrate characteristics, and operational conditions such as temperature, HRT, SRT are important parameters for efficient treatment (Mao et al., 2015; Siddique and Wahid, 2018). In LtAD systems, slow growth of microbes and reduced microbial activity suggest inoculum characteristics are a crucial factor (McKeown et al., 2012; Pavlostathis and Giraldo-Gomez, 1991). One challenge for LtAD processes is obtaining inoculum which has microbial consortium needed to match a treatment scenario, since most AD reactors currently operate at mesophilic conditions. Due to the easier availability of mesophilic inoculum, they have been used in LtAD of municipal and industrial wastewaters (El-Kamah et al., 2010; Enright et al., 2009; Fia et al., 2012; Keating et al., 2018; McHugh et al., 2006; Sheldon and Erdogan, 2016; Singh et al., 2019b, 2019a; Trzcinski and Stuckey, 2010; Zhang et al., 2012). These studies suggested that the enrichment of specific taxa during reactor operation is subject to not only the initial composition of the inoculum and resultant *a priori* effects, but also the operational conditions such as temperature, for example, development of psychrophilic anaerobic microbial consortium in hybrid reactors treating acidified wastewaters needed a long duration (1243 days) (McKeown et al., 2009). Moreover, microbial composition of most abundant taxa from low temperature studies has similar abundance profiles suggesting a prominent role of temperature (Trego et al., 2021). Knowledge regarding LtAD of wastewaters is limited for recalcitrant wastewaters rich in FOG and/or LCFA. Therefore, the general applicability of inoculation approaches for psychrophilic anaerobic digestion should be ascertained through empirical testing due to inherent instability of performance in these systems (Tiwari et al., 2021; Yao et al., 2020).

LtAD systems are complex, involving interactions between multiple bacterial groups and the archaea to ultimately degrade an organic substrate to methane. Core microbiomes are composed of shared amplicon sequence variants (ASVs) that are consistently present at all tested conditions and are likely to participate in basic metabolic processes in a niche. This concept has been widely applied to understand composition of microbial communities involved in metabolic processes of anaerobic digesters (Mei et al., 2016; Peces et al., 2018; Xu et al., 2018). We have previously deduced the active core microbiomes from anaerobic dynamic sludge chamber fixed film reactors treating LCFA-rich wastewaters (Singh et al., 2022), however, the presence of a 'collated core microbiome' in LtAD reactors fed with similar wastewaters is yet unknown and needs attention. Moreover, instability in the microbial interactions in AD systems can lead to inhibition of syntrophic bacteria and the methanogens which ultimately can cause a buildup of acids and reactor acidification (Maurus et al., 2021). Correspondingly, despite the robustness and stability of AD processes, there is limited knowledge regarding the complexity of the microbial community at different operational conditions (Cabezas et al., 2015). In full-scale AD systems treating brewery wastewater, methanogenic activity correlated with microbial community evenness at any time demonstrating a strong relationship between community structure and its function than its environment (Werner et al., 2011; Wittebolle et al., 2009), due to the higher capacity of microbial consortia to use redundant functional pathways conferring robustness and resilience to the microbial community (Carballa et al., 2015). Recently, the importance of microbial interaction networks linking to their process performance at fluctuating operational conditions has been highlighted for improved understanding of the stability of AD process (Saha et al., 2020). Fed-batch reactors operated with varying frequencies of repeated organic load shocks (at every 2, 4, 6, and 8 days),

showed deteriorated methane productivity and a loss in network connectivity at the least frequent organic shock loads (8 day-intervals) (Mercado et al., 2022), suggesting a breakdown in microbial interactions for stable AD process. Even in stable mesophilic AD reactors, correlational network analyses showed microbial interactions demonstrate successional patterns such as decreased network complexity (Wu et al., 2016). Hence, the stability of this complex microbial network is crucial for optimal functioning of AD systems and understanding the relationships between complexity and stability in microbial interaction networks may aid in optimizing the anaerobic treatment performance of the LtAD systems. Typically, interactions between taxa are generated using co-occurrence or interaction network analyses (Xu et al., 2020; Zhu et al., 2021). However, the accurate representation of microbial networks requires datasets having multiple replicates for each variable in order to achieve strength in the obtained relationships between taxa (Hugerth and Andersson, 2017). Furthermore, any 'causal relationships' arising from the analytical biases in the experimental and sampling methodology or analyses may deviate the network outcomes. Hence, it is important to assess if interactions between taxa are stable, thereby inferring the stability of the constructed network. Complexity-stability relationship defines the relationships in a group by evaluating how two species are connected (Yonatan et al., 2022). A change in species abundance reflects a change in species interactions and can be assessed by evaluating the topology of inferred network wherein a high connectance suggests that a local perturbation in the abundance of one or a few species is expected to propagate and affect the entire community much more substantially than a system with low effective connectance. Evaluation of complexity-stability relationship in LtAD systems treating LCFA-rich wastewater will help indicate the stable microbial niches that underpin process stability in such systems.

Accordingly, this study examined the bacterial and archaeal community diversity (alpha and beta) and core microbiomes from 106 16S rRNA datasets that were obtained from research studies investigating LCFA treatment in LtAD systems. Furthermore, various factors were assessed including: inoculum, substrate, operational, reactor operation mode and reactor configuration, and the type of nucleic acid sequenced, for their role in shaping the microbial community diversity (richness, evenness and beta-diversity). Based on the key driving factors, microbial clusters were determined from the collated dataset. The novelty of this work is that, for the first time, the microbial community diversity and core microbiomes were deduced from collated datasets from LtAD systems undertaking LCFA degradation. Environmental fitting of the clusters, their correlational networks and stability of the correlational networks were assessed, to obtain insights into the factors driving the microbial community dynamics of anaerobic LCFA degradation at low temperatures.

2. Methods

2.1. Data collection and description

Publicly available 16S rRNA microbial community sequencing datasets on anaerobic LCFA degradation at 10 and 20 °C were retrieved along with their operational and experimental data. Selection of the 16S rRNA datasets was limited to samples sequenced on Illumina platform to avoid systematic biases and enabling higher quantitative power. In this study, 16S rRNA sequences were obtained from 106 samples from three peer-reviewed publications featuring publicly available data from NCBI sequence read archive (SRA) (https://www.ncbi.nlm.nih.gov/sra). Metadata was extracted for inoculum characteristics (inoculum source, inoculum morphology), substrate characteristics (type of substrate), operational characteristics (loading rate, operational temperature).

The datasets included (Fig. 1):

Study 1 (Singh et al., 2019a) – Three different mesophilic inocula (two municipal digestates – Viniikanlahti Digestate (VD), and Rauhola Digestate (RD), and one granular sludge (GS)) from Finland were assessed for methane production from LCFA-rich synthetic wastewater at 10 and 20 °C in batch assays in a 200-d batch incubation. The three mesophilic inocula were sourced from reactors originally operated at 33–37 °C. Sixty 16S rRNA sequences (SRA: SRP164945) and the associated metadata generated during study were used for analysis in current study.

Study 2 (Singh et al., 2019b) – A mesophilic granular sludge (GS) from Finland was assessed for methane production from LCFA-rich synthetic wastewater at 20 °C in EGSB reactors operated in continuous mode. The diversity and dynamics of microbial community in the GS were monitored at 98-day operational duration at hydraulic retention times (HRT) of 24 and 18 h. Twenty-one 16S rRNA sequences (SRA: SRP164945) and the associated metadata generated during study 2 were used for analysis in current study.

Study 3 (Singh et al., 2022) – Anaerobic flocculent sludge treating FOG-containing dairy effluent at mesophilic conditions was combined with an anaerobic granular sludge treating dairy wastewater at ambient conditions, and used as '*mixed inoculum*' (Singh et al., 2020). This mixed inoculum was assessed for methane production from LCFA-rich synthetic wastewater at 20 °C in continuously operated dynamic sludge chamber – fixed film (DSC-FF) reactors at HRTs of 72, 42.5, 24, 18 and 12 h. The diversity and dynamics of microbial community in the bottom granular sludge layer (from DSC) and biofilm layer (in FF) were monitored temporally during a 150-d operational duration (Singh et al., 2022). Twenty-seven 16S rRNA sequences (SRA: PRJNA657615), 15 from granules (from DSC) and 12 from the biofilm (from FF), and the associated metadata, generated during study 3 were used for analysis in current study.

Studies 1, 2 and 3 were sequenced on an Illumina MiSeq platform using the V3-V4 hypervariable regions and the universal bacterial/archaeal primer sets of 515f-806r (Caporaso et al., 2012). Meta-analysis of anaerobic LCFA-degrading microbiomes was performed to elucidate the factors that differentiate microbial communities, the factors being:

- inoculum characteristics (inoculum source (RD, VD, GS, mix), and, inoculum morphology (suspended, granular, mixed)),
- substrate characteristics (substrate type - no substrate, acetate, LCFA-rich dairy, and LCFA percentage (COD basis) in feed), LCFA mixture in the studies 1–3 contained palmitate, stearate, oleate and linoleate in a ratio of 30:15:45:10 on COD basis. Palmitate and stearate are saturated LCFAs, whereas oleate and linoleate are unsaturated LCFAs, and were used to simulate complex LCFA-rich wastewaters.
- operational characteristics (organic loading rate (gCOD/L for batch and gCOD/L·d for continuous reactors), and operational temperature (10 °C, 20 °C)),
- reactor operation mode (batch or continuous), and reactor configuration (batch digester, EGSB, DSC-FF), or,
- the type of nucleic acid sequenced (total vs active community).

The categorical and numerical data associated with the different sample groups in the studies 1,2, and 3 are listed in Supplementary Table 1.

2.2. Bioinformatics

We processed each dataset separately, using open-source bioinformatics pipeline QIIME2 (Bolyen et al., 2019). Initially, the 2 × 300 bp paired-end Illumina Miseq sequences were demultiplexed and quality trimmed using Phred quality score of 20 giving an amplicon length of 291 bp. Deblur Algorithm within QIIME2 was then employed to recover ASVs (Amir et al., 2017). All three studies generated independent BIOM files, with the summary statistics as follows:

Study 1 (n = 60 samples, p = 132,855 ASVs) with summary statistics of reads per samples as [1st Quartile: 31,096; Median: 39,426; Mean: 42,679; 3rd Quartile: 45,342; Max: 404,084]

Study 2 (n = 21 samples, p = 24,890 ASVs) with summary statistics of reads per samples as [1st Quartile: 33,783; Median: 39,656; Mean: 45,573; 3rd Quartile: 49,517; Max: 134,193]

Study 3 (n = 44 samples, p = 75,972 ASVs) with summary statistics of reads per samples as [1st Quartile: 116,199; Median: 130,783; Mean: 129,344; 3rd Quartile: 147,509; Max: 206,969]

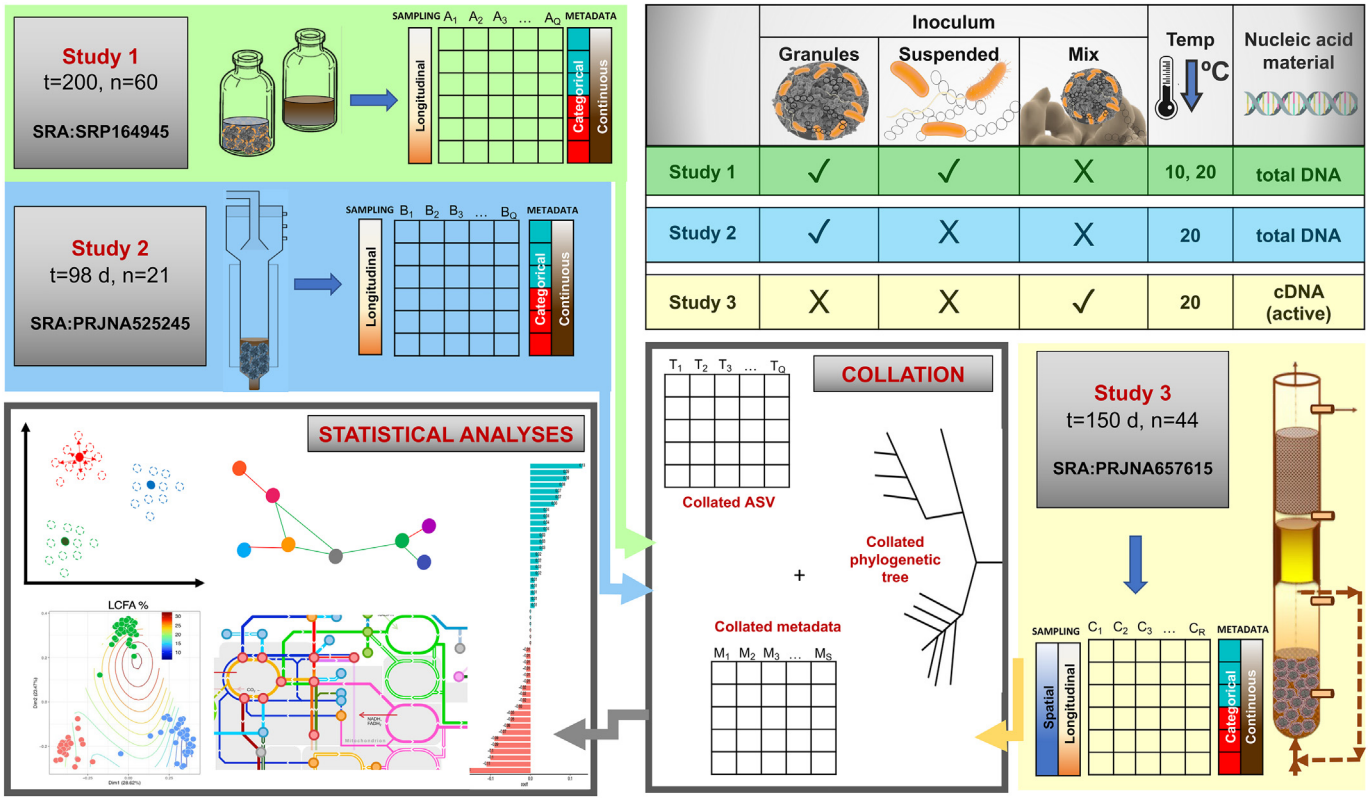

Fig. 1. Schematic representation of the datasets, methodological steps, collation workflow and statistical analyses employed in this study.

To combine these studies together, we have used our recently published collation strategy for comparative analysis (Keating et al., 2020; Mills et al., 2022; Thom et al., 2022). Briefly, the 16S rRNA sequences from multiple studies were combined by finding the species that are shared between the studies. It was achieved by matching ASVs from each study to the reference database and collating these matched ASVs into a single biom file. The sequences without sequence- level assignments were removed. We found from the previous studies that the resolution of species taxonomy is highly dependent on the choice of database, amplicon V-region, and the type of classifier, which we explored in Keating et al. (2020). Our workflow relied on utilizing a newer "Bayesian Lowest Common Ancestor (BLCA)" algorithm (Gao et al., 2017) as opposed to "Naïve Bayes Classifier" (used in QIIME2) to align the ASVs against the reference database. SILVA SSU Ref NR release v138 was the reference database used (Quast et al., 2013). BLCA approach resolves more ASVs at sequence level resolution, a requirement to recover as many full-length 16S rRNA sequences, reduce data loss, and preserve beta diversity between samples after collation. The limitation of meta-analyses is read loss, either due to use of inefficient classifier, or a lack of sequences availability in the reference database to train the classifier. As can be seen in Supplementary Figs. S1–S3, the overall beta diversity patterns between the uncollated and collated datasets were largely preserved as shown by Mantel analyses. After collation, the summary statistics were: collated (n = 125 samples, p = 9,950 ASVs) with summary statistics of reads per samples as [1st Quartile: 34,717; Median: 45,219; Mean: 73,416; 3rd Quartile: 118,898; Max: 402,853]. Out of the total of n = 125 samples, we retained only reactor biomass samples (n = 108 samples), a further 2 samples were removed after performing additional quality checks (removing Chloroplasts and Mitochondria and any samples <5000 reads) leaving a total of 106 samples with 4661 full-length ASVs.

Using the full-length 16S rRNA ASVs within QIIME2, the q2-alignment method MAFFT (Katoh and Standley, 2013) was used to create multi-sequence alignment of ASVs, and afterwards a mask is applied to remove phylogenetically ambiguous alignments to obtain rooted phylogenetic tree using FastTree (Price et al., 2010) within q2-phylogeny framework.

2.3. Statistical analyses

The collated abundance table with taxonomy, phylogenetic tree, and metadata was processed using the microbiome seq packages in R. The R scripts used for statistical analysis are available at http://userweb.eng.gla.ac.uk/umer.ijaz/bioinformatics/ecological.html (accessed on 1 January 2023) and R's microbiomeSeq package accessible at http://www.github.com/umerijaz/microbiomeSeq (accessed on 1 January 2023).

Alpha diversity metrices. Statistical analyses were performed in R using the tables generated as above and metadata associated with the study. The Vegan package (Oksanen et al., 2016) was used for the analysis of alpha and beta diversity in microbial samples. For alpha diversity, the indices used were: (i) rarefied richness – to represent the estimated number of species in a rarefied sample (to minimum library size); (ii) Shannon entropy – to represent a measure of balance within a community. R's aov() function was used to calculate the pair-wise analysis of variance (ANOVA) p-values which were then drawn on top of alpha diversity figures.

Beta diversity. For beta diversity, the dissimilarity in species community composition between pairwise comparisons of bacterial communities were represented in Principal Coordinate Analysis (PCoA) ordination plots by calculating two different distance metrices using Vegan's cmdscale() function: (i) Bray Curtis, which considers the species abundance count; and (ii) Unweighted Unifrac, which considers the phylogenetic distance between the branch lengths of ASVs observed in different samples, calculated using the phyloseq package (McMurdie and Holmes, 2013).

Core microbiome and differential heat trees. The core microbiome analysis for each of the clusters obtained from collated dataset was carried out, considering a prevalence of 95 % for all ASVs (Lahti et al., 2019) was determined using the library microbiome (Shetty et al., 2017). Differentially expressed clades in the clusters obtained from collated dataset were visualized as heat trees (Foster et al., 2017) (using adjusted Wilcoxin p-value test) in the library metacoder.

Environmental Fitting. To see if changes in covariates (LCFA % and temperature) have an impact on microbial community structure, we fitted smooth surfaces of the covariates on ordination plot (PCoA in this case) using penalized splines by employing ordisurf() function from R's Vegan package (Oksanen et al., 2016). The method uses generalised additive model (GAM) by regressing the covariate as C ~ S (Dim1, Dim2), where Dim1 and Dim2 are the ordination scores extracted from PCoA and S () is a spline function. The environmental variables - LCFA % and temperature fitted well i.e., $p < 0.05$.

CODA-LASSO Model. In presence of a relationship between the environmental variable and microbial taxa abundance, regression fitting was performed using CODA-LASSO model. To see the relationship between environmental covariates (LCFA % and temperature) and the minimal subset of microbes that can explain them, we used the variable selection approach where through penalized regression on the set of all pairwise log-ratios (Susin et al., 2020) we identified two disjoint subsets of microbes, those that are positively associated, and those that are negatively associated with the covariate of interest. Briefly, we used the CODA-LASSO approach (Lu et al., 2019) where the abundance of individual covariate y_i (LCFA % or temperature) is modeled as $y_i = \beta_0 + \beta_1 \log(x_{1i}) + \ldots + \beta_j \log(x_{ji}) + \epsilon_i$ (for i-th sample and j-th species, with x_{ji} being the microbe abundance) with the constraint $\sum_{k\geq 1}\beta_k = 0$ (i.e., all β-coefficients sum up to 1), and these regression coefficients $\boldsymbol{\beta} = (\beta_0, \ldots, \beta_j)$ are estimated to minimise $\Sigma_{i=1}^{n}(y_i - \beta_0 - \beta_1 \log(x_{1i}) - \ldots - \beta_j \log(x_{ji}))^2 + \lambda\Sigma_{k\geq 1}|\beta_k|$ subject to $\Sigma_{k\geq 1}\beta_k = 0$ (using a soft thresholding and projection algorithm) for n samples. Here, λ is the penalization parameter in LASSO shrinkage terms $\lambda\Sigma_{k\geq 1}|\beta_k|$ which forces some of the $\boldsymbol{\beta}$-coefficients to go zero, particularly those that do not have a relationship with the covariates and serves as a means to do variable selection. The non-zero $\boldsymbol{\beta}$-coefficients are then divided into two groups, those that are positively associated with the environmental covariate, and those that are negatively associated with the environmental covariate, respectively. For this purpose, we used coda_glmnet() function from R's coda4microbiome package (Calle and Susin, 2022). We have used the top 100 most abundant genera in the CODA-LASSO model.

Generalised Linear Latent Variable Model. To find the relationship between microbial communities and sources of variation (*inoculum source, inoculum characteristics, temperature, substrate, nucleic acid type* whether *DNA or cDNA, reactor configuration, LCFA %, organic loading rate (OLR)*), we have used Generalised Linear Latent Variable Model (GLLVM) (Niku et al., 2019), which extends the basic generalised linear model that regresses the mean abundances μ_{ij} (for i-th sample and j-th microbe) of individual microbes against environmental covariates x_i as above by incorporating latent variables u_i as $g(\mu_{ij}) = \eta_{ij} = \alpha_i + \beta_{0j} + \boldsymbol{x}_i^T\boldsymbol{\beta}_j + \boldsymbol{u}_i^T\boldsymbol{\theta}_j$, where $\boldsymbol{\beta}_j$ are the microbe specific coefficients associated with individual covariate (a 95 % confidence interval of these whether positive or negative, and not crossing 0 boundary gives directionality with the interpretation that an increase or decrease in that particular covariate causes an increase or decrease in the abundance of the microbe), and $\boldsymbol{\theta}_j$ are the corresponding coefficients associated with latent variable. β_{0j} are microbe-specific intercepts, while α_i are optional sample effects which can either be chosen as fixed effects or random effects. To model the distribution of individual microbes, we have used Negative Binomial distribution. Additionally, the approximation to the log-likelihood is done through Laplace approximation (LA) with final sets of parameters in glvmm() function being family = 'negative.binomial', method = "LA", and control.start = list (n.init = 5, jitter.var = 0.1) that seemed to fit well. This, we did for top 100 most abundant genera in our datasets. In addition, the factor loadings $\boldsymbol{\theta}_j$ store correlations of microbes with the residual covariance matrix $\boldsymbol{\Sigma} = \boldsymbol{\Gamma}\boldsymbol{\Gamma}^T$ where $\boldsymbol{\Gamma} = [\theta_1 \ldots \theta_m]$ for m latent variables. This residual covariance matrix gave co-occurrence relationship between microbes that are not explained by environmental covariates as above.

Complexity-Stability relationships. To understand complexity-stability relationship in our dataset, we have estimated the effective connectance D^2 after fitting a regression model to samples overlap in terms of species they share and the sample dissimilarities (Yonatan et al. (2022). This precludes the need to infer co-occurrence relationship explicitly, leading to D^2 serving as a proxy for stability. As per author's recommendation, D^2 was obtained by the slope of regression fitted to the dissimilarity-overlap plot to the 25 % top overlap values for the paired-wise dissimilarity/overlap values for N samples in a given category (suspended, mix, or granules) from a total of $N(N-1)/2$ paired-wise values.

3. Results and discussion

3.1. Statistical distribution of the samples

A high correlation (Mantel statistic: $R^2 \geq 0.96$; $p < 0.01$) between the original full ASV table and the reduced ASV table in the collated dataset obtained for each study. This indicated a minimal loss of beta diversity even with the removal of ASVs which were not present in the reference database. Application of BLCA approach has shown to resolve ASVs at sequence level resolution, and reduce data loss between samples after collation in microbiomes from other engineered systems such as microbial electrosynthesis (Mills et al., 2022) and drinking water treatment systems (Thom et al., 2022). The 25 most abundant classes are shown in Figs. S1–S3 for individual and collated datasets to provide visual cues in terms of how similar the datasets are after filtering out ASVs. Among the top 25 most abundant classes, the archaeal classes *Methanobacteria* and *Methanosarcinia* and the bacterial classes *Aminicenantia*, *Caldatribacteria*, *Bacilli*, *Gammaproteobacteria*, *Synergistia* and *Clostridia* were observed in all the collated datasets from the three studies.

3.2. Diversity of anaerobic LCFA-degrading microbial communities

We assessed the impact of factors - inoculum source and morphology, reactor operation mode and nucleic acid type on the microbial diversity (richness and evenness) of LCFA-degrading microbial communities in LtAD systems. Our datasets included four inocula sources – RD, VD, GS and mixed source (mix); which differed in their morphologies and designated as 'suspended' (for RD and VD), 'granular' (for GS), and 'mix' (for the mixed sludge source) (Fig. 2A). These inocula were seeded in anaerobic reactors that were operated in batch or continuous-mode and sampled on different days with subsequent extraction and sequencing of either the whole DNA (representing entire microbial community), or the cDNA (representing the active community).

Microbial diversity – richness and evenness (Fig. 2A) differed in the sample groups based on the factors - inoculum source and morphology, nucleic acid type and reactor operation mode ($p < 0.001$) (Fig. 2A). Microbial diversity for sample groups closely followed their seed, based on the inoculum source and morphology. Reactors seeded with suspended sludges (RD and VD) had similar microbial diversity during the 200-d experimental duration, despite being fed with different substrates (no substrate, acetate, or dairy wastewater) (Fig. 2A). The reactors with mixed sludge (mix) had similar richness and evenness ($p > 0.001$) compared to the seed populations, even after continuous operation in DSC-FF reactors for a period of 150 days. In comparison, in the EGSB reactors seeded with granular sludge, microbial richness decreased during continuous operation (marked by symbol +) but the richness did not further change (Fig. 2A). During continuous flow operation of EGSB, high levels of LCFA in feed contribute to the loss in granular sludge integrity which results in washout. The lack of retention of washed out microbes in the EGSB reactors likely resulted in the reduction of species richness of the bioreactor and deteriorated performance for anaerobic LCFA degradation (Singh et al., 2019b). In comparison, the suspended sludges when fed with identical LCFA-rich wastewater were in closed batch systems, where the risk of washout was substantially reduced. Taken together, these new insights suggest inoculum characteristics, reactor configuration and operation structuring the abundance and diversity of anaerobic sludges.

Diversity between the samples (beta diversity) was displayed through PCoA plots explained 52.2 % of the variation in abundances, and 41.11 % of the variations in phylogeny (Fig. 2B). The samples were grouped into

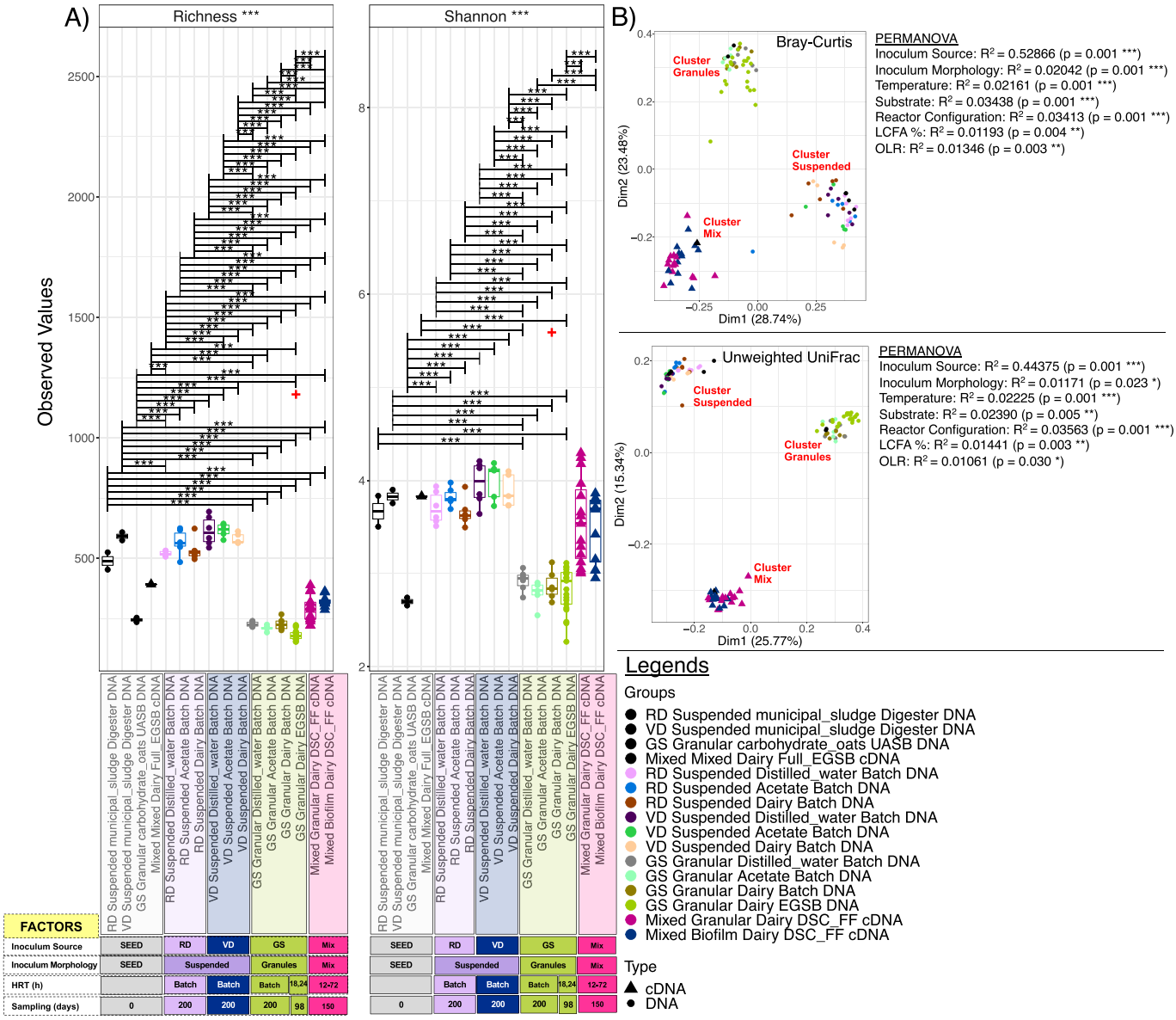

Fig. 2. Diversity in the microbiomes of the inocula and reactor samples collated in this study, categorized by combination of different factors - inoculum characteristics, substrate characteristics, reactor type and nucleic acid type. (A) Alpha diversity box plot in inocula and samples represented by rarefied richness and Shannon evenness. Samples are grouped based on inoculum source used as seed. PERMANOVA explains significant variability in microbial community structure from different bioreactor compartments and at different HRTs. Lines for panels A, B, C and D connect two sample groups at statistically significant levels indicated by asterisks as * ($p < 0.05$), **($p < 0.01$) or ***($p < 0.001$). (B) Beta diversity in inocula and samples represented through principal coordinate analysis (PCoA) plots calculated using Bray-Curtis and unweighted UniFrac distances.

three distinct clusters on the PCoA plot when using composition (Bray-Curtis distances) and their phylogeny (UniFrac distances). The factor that significantly contributed to the beta diversity was primarily the inoculum source (R^2 = 0.44–0.52, $p < 0.001$), with no overlap between 'granular', 'suspended' and 'mix' clusters. To a substantially lower extent, the inoculum morphology, operational temperature, substrate characteristics, reactor configuration and operational parameters (LCFA percentage (LCFA%), OLR) contributed to the variability in the observed beta diversity (R^2 = 0.01–0.03, $p < 0.1$).

The results presented herein suggest the seed microbial community diversity continues to exert influence on the microbial community diversity and this effect is still pronounced during periods of long-term operation in both open reactor systems (up to 150 d) and closed systems (up to 200 d), irrespective of operating conditions or substrate. Anaerobic consortia have optimum growth at mesophilic temperatures 30–37 °C (Pommerville, 2014). At low temperatures (≤ 20 °C), the substrate uptake rate and microbial growth kinetics decrease (Nedwell, 1999; Singh et al., 2019a). The syntrophic bacteria and methanogenic archaea have long doubling times. For example, at 35–37 °C the methanogenic genera *Methanosaeta* and *Methanobacteria* have doubling times of 4–9 d and 6–35 h respectively, whereas the syntrophic bacterial genera, *Syntrophomonas* and *Syntrophus* have doubling times of 11–50 h. According to the Arrhenius model, the doubling times become considerably longer (~2-times) with every 10 °C decrease in temperature, which means the doubling time for LCFA-degrading syntrophic bacteria would increase to 30–154 h at 20 °C, and 62–318 h at 10 °C. Therefore, longer microbial retention times are needed to maintain treatment efficiency. Moreover, abundances of these syntrophic bacteria are lower than that of fermentative bacteria and methanogenic archaea in LtAD systems treating LCFA (Singh et al., 2022). Additionally, methane is the terminal product of AD process, and has lower Gibbs energy change per electron than the other organic intermediates such as VFAs and carbon monomers (Kleerebezem et al.,

2015), thus methanogenesis driving the AD process. Hence, inoculum should be selected with consideration for high β-oxidation and methanogenesis activity in LtAD systems treating LCFAs, as the initial concentrations and activities of syntrophic bacteria and methanogens in the seed inoculum will influence the LCFA degradation potential.

3.3. Core microbiome of anaerobic LCFA-degrading microbial communities

In LtAD systems methanization requires syntrophy between bacterial and archaeal groups to undertake successive hydrolysis, acidogenesis, acetogenesis and methanogenesis. We obtained the core microbiomes in the three clusters at a very high prevalence (>95 %). Core bacterial microbiomes in the clusters 'suspended' and 'mix' was complex involving a high number of taxa. We identified the core bacterial taxa in microbiomes of LCFA-degrading communities in AD, ranging from 5476 in cluster 'suspended', 3400 in cluster 'mix' and 2075 in cluster 'granules'. In comparison, fewer archaeal taxa constituted the core microbiome, comprising of 7, 7 and 3 taxa in the clusters 'suspended', 'mix' and 'granules' respectively. Across the three clusters, the prevalent methanogenic archaeal genera were *Methanosaeta* and *Methanobacteria*, whereas the prevalent bacterial genera were *Longilinea*, *Christensenellaceae R7 group*, *Lactivibrio*, *candidatus Caldatribacterium*, RBG − 13 − 54 − 9 from family *Anaerolineae*, *Aminicenantales* and an uncultured taxon from *Propionibacteriaceae* family (Fig. 3). These core bacterial genera are known to have fermentative

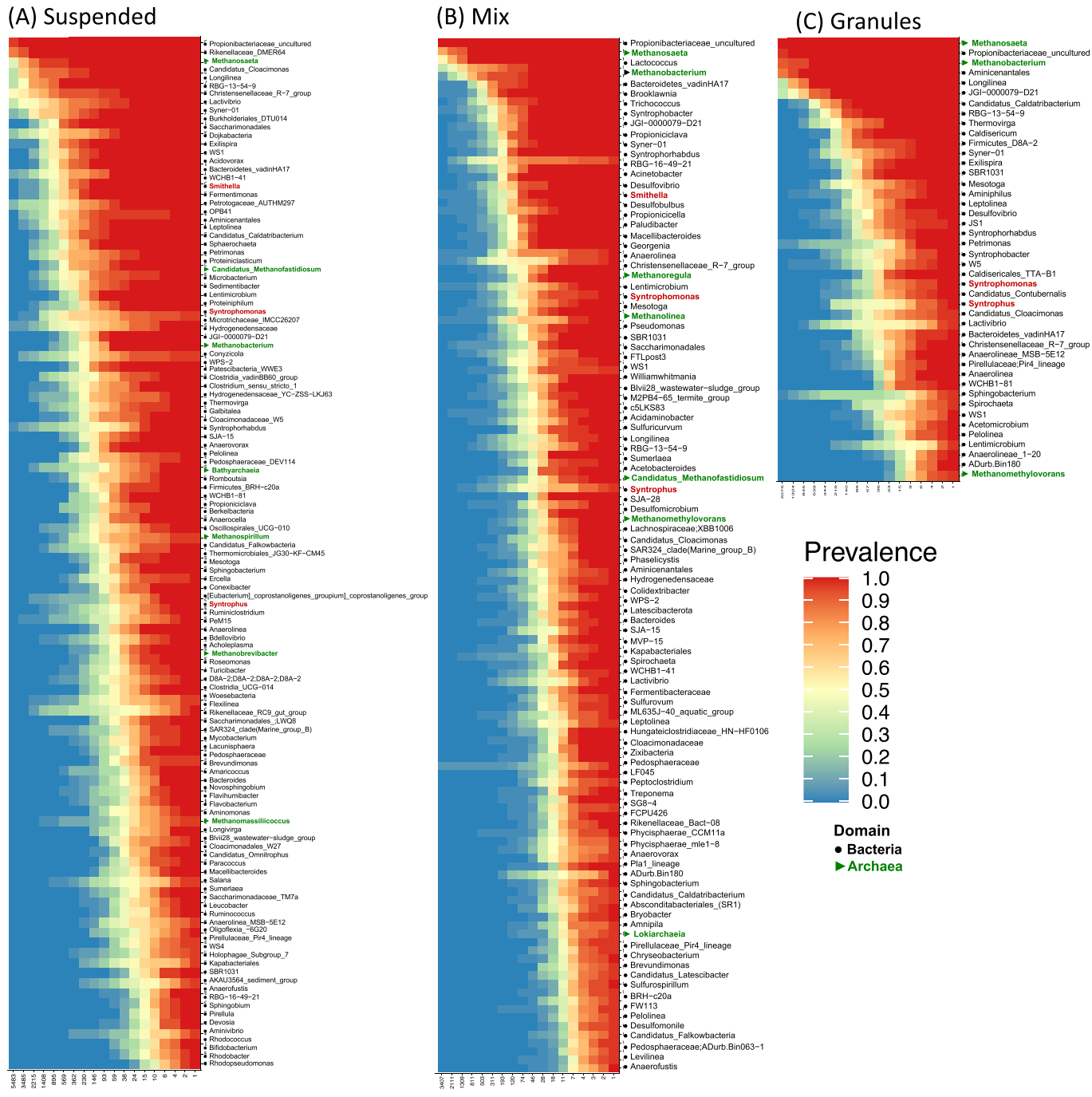

Fig. 3. Core microbiome analysis: heatmaps of the different meta-sample groups derived from this study (A) Cluster 1, corresponding to suspended sludge samples 'suspended', (B) Cluster 2 corresponding to mixed inocula samples 'mix', (C) Cluster 3 corresponding to granular sludge samples 'granular'. Minimum prevalence was set at 0.95 for all groups.

metabolism and thus, were likely involved in acidogenesis and acetogenesis steps of anaerobic digestion (Dodsworth et al., 2013; Kadnikov et al., 2019; Yamada et al., 2007). However, little information is available for the uncultured and candidatus taxa and RBG-13-54-9, which form part of microbial dark matter (i.e., the microbes that have not yet been obtained in pure culture, due to either lack of knowledge or inability to supply the required growth conditions). Such candidatus taxa are well characterized by genetic sequences but yet remain uncultured due to slow growth rate, or a lack of knowledge of optimum growth conditions and taxa interdependence (Liu et al., 2022). An expansion of knowledge base for such obscure LCFA-degrading bacteria will aid in understanding their role in the LtAD process, and lead to possible exploitation for bioengineering applications.

LCFAs – unsaturated (linoleate, oleate), as well as saturated (stearate and palmitate) are known to be anaerobically degraded by 4 bacterial species – *Syntrophomonas sapovorans, Syntrophomonas curvata, Syntrophomonas zehnderi, and Thermosyntropha lipolytica* to medium chain and short chain fatty acids. In addition, *Syntrophus aciditrophicus* can degrade the saturated LCFAs (palmitate and stearate) to medium and short chain fatty acids (Sousa et al., 2009). Hence, monitoring these species is important for estimating the overall LCFA degradation potential. In this study, the known syntrophic LCFA degraders – *Syntrophus* and *Syntrophomonas* were present in core microbiomes of all three clusters, while *Smithella* was present in clusters 'mix' and 'granular' but not in the cluster 'suspended' (Fig. 3). The prevalence of abovementioned bacteria and archaea in the core microbiomes of different clusters, signifies that they occupy a range of niches and putative trophic roles in the low temperature anaerobic treatment of LCFAs. It is likely the prevalent fermentative bacteria represented in core microbiomes hydrolyzed the LCFA-rich substrates to produce shorter chain fatty acids which were used by the syntrophic LCFA degraders to produce VFAs including acetate, and also hydrogen. The acetate and hydrogen were converted to methane through acetoclastic and hydrogenotrophic methanogenesis by genera *Methanosaeta* and *Methanobacterium*, respectively. While both acetoclastic and hydrogenotrophic pathways contribute to methanogenesis during low temperature AD (Keating et al., 2018; Trego et al., 2021), the dominant methanogenesis pathway at sub-mesophilic temperatures remains the subject of debate and further work is recommended. From our analysis of 106 sequences obtained from anaerobic LCFA-fed reactors seeded with diverse inocula, we found *Methanosaeta* as the most prevalent archaea across the datasets suggesting a crucial role of *Methanosaeta*-mediated acetoclastic methanogenesis in anaerobic low temperature (≤ 20 °C) LCFA degradation.

Broadly, establishment of methanogenic pathway in anaerobic systems is affected by the kinetics and thermodynamics of the system and concentrations of toxicants in the system. However, methanogenesis is the terminal step in the AD process, and concentration profiles of the methanogenic precursors, specifically acetate and hydrogen rely on their concentration in the influent substrate and their generation during the AD trophic stages of hydrolysis, acidogenesis and acetogenesis. Hence, substrate characteristics will affect establishment of methanogenic pathway in anaerobic systems. For example, an acetate-rich influent will promote prevalence of acetate-utilizing methanogens such as *Methanosaeta* or *Methanosarcina*. Additionally, high hydrogen concentrations produced during fermentation of substrate will promote prevalence of hydrogen utilizing methanogens such as *Methanobacterium* or *Methanosarcina*. Conventionally it has been accepted that *Methanosaeta* outcompete *Methanosarcina* at acetate concentrations lower than 1 mM due to their higher substrate affinity (Conklin et al., 2006), but *Methanosaeta* has been shown to outcompete *Methanosarcina* at higher acetate concentration (>20 mM) (Chen and He, 2015) due to divergent uncharacterized populations of *Methanosaeta* demonstrating high acetotrophic activity. At low ambient and psychrophilic temperatures, hydrogenotrophic methanogenesis is thermodynamically more feasible compared to acetoclastic methanogenesis. In this meta-analysis, *Methanosaeta* was the only acetotrophic methanogen resolved from Silva database whereas *Methanosarcina* was not found. The substrates used in current study were rich in LCFAs and/or skim milk powder (rich in lactose), both of which produce acetate. Each round of β-oxidation of LCFAs produces acetate and hydrogen, additionally, acetate is produced from lactose. The prevailing metabolic environment in the EGSB and DSC-FF reactors consisted of acetate (10–75 mg/L) evidencing their production from the LCFA-rich wastewaters, which promoted the growth of *Methanosaeta* (Singh et al., 2020, 2019b).

Kinetics in a AD process refers to substrate degradation kinetics and microbial activity, wherein the substrate degradation kinetics is affected by its solubilization and uptake by the microbial consortia. An acclimatized microbial consortium aids in high balanced metabolic activity of hydrolysers, acidogens, acetogens and methanogens, aiding in optimal substrate transfer across the AD trophic groups. In comparison to mesophilic and thermophilic conditions, the substrate kinetics at low ambient or psychrophilic conditions such as 10–20 °C, is affected by reduced substrate availability to the microbes and the reduced microbial activity at lower temperatures. The prevailing concentrations of methanogenic precursors, acetate and hydrogen, structure the dominance of specific methanogenic archaea in LCFA-rich LtAD systems. Additionally, gaseous diffusion and viscosity increase at low temperatures (Lettinga et al., 2001), thereby altering the mixing and fluid movement pattern and imposing higher energy requirements for mixing the solid (substrate and microbes), liquid (wastewater) and gaseous (methane, hydrogen, carbon dioxide, hydrogen sulphide) phases (Eshtiaghi et al., 2013). Recent advances in novel reactor designs enable packing of larger amounts of active methanogenic consortia in reactor (e.g., membrane bioreactors, biofilm reactors) (Månsson, 2020; Szabo-Corbacho et al., 2021; Wusiman, 2021) and may aid in improving kinetics of LCFA degradation and microbial activity LtAD in these systems. To the best of authors' knowledge, hydrodynamic parameters have not yet been investigated for LtAD of LCFAs, particularly with an emphasis on engineering the microbial consortia, thus, the impact of these parameters on establishment of methanogenic pathway is not validated in this study.

Heat tree analysis revealed that the prevalence of certain taxa varied among the three clusters (Supplementary Fig. 4). The Wilcox tests were performed on the relative abundances of taxa, and the log ratio of means were drawn when the adjusted *p*-values were significant after correcting for multiple comparisons to determine the differentially abundant taxa. Abundances of taxa belonging to *Alphaproteobacteria* and *Bacteroidia* (particularly *Bacteroidales*) differed among the clusters following the trend: suspended > mix > granular. *Syntrophomonas* abundances followed the trend: suspended > granular > mix suggesting its prevalence in suspended sludges, whereas the opposite trend of *Geobacteraceae* abundances (mix > granular > suspended) suggests an important active role in LtAD. *Geobacteraceae* taxa are important in degradation of palmitate and oleate at 35–37 °C (Cavaleiro et al., 2020; Hatamoto et al., 2007), due to their potential for LCFA degradation as indicated by presence of *long-chain fatty acyl-CoA dehydrogenase* expressing *fad E* gene (Aklujkar et al., 2010). Concurrently, *Geobacter* in syntrophy with *Syntrophomonas* can scavenge hydrogen faster than *Methanobacterium* (Cavaleiro et al., 2020), driving the *Syntrophomonas*-mediated LCFA degradation. Many taxa found in the core microbiomes were uncultured or at candidatus status, representing a large abundance of microbial dark matter in LtAD systems. There is a need for understanding the roles of persistent taxa that form the microbial dark matter for improved comprehension of microbial dynamics especially during scale up of this technology, and for energy recovery from FOG and LCFA-rich wastewaters at low temperatures. The bacterial genera identified from the core microbiomes and heat tree analyses in this study may have a significant role in LtAD of LCFA-rich wastewaters at 10–20 °C. Future work should validate the roles of key taxa by isolating pure cultures and stable isotope probe labelled-metagenomics.

3.4. Relationship of environmental variables to microbial community dynamics

Differences in the microbial communities based on their operational conditions (continuous environmental variables of interest – temperature, LCFA%, OLR) were explored by using GAM models and represented as beta diversity contour plots. Before fitting a regression model, such as CODA-LASSO, it is recommended to employ environmental fitting to

investigate existence of a relationship. GAM models are employed to regress the environmental variable (continuous) against the scores in reduced order representation (i.e., scores on dimension 1 and dimension 2), and if the regression is a perfect fit i.e., GAM $p < 0.05$ then there is a probable relationship between environmental variable and the associated microbiome.

The beta diversity contour plot with LCFA% values regressed against the scores of the ordination (PCoA) showed that the three clusters (granular, mix, suspended) were distinctly apart despite treating LCFA-rich wastewaters with variations only in their LCFA percentage (Fig. 4A). Since the regression was a perfect fit ($R^2 = 0.98$, $p < 0.05$), it showed a relationship between LCFA% and the associated microbiomes (Fig. 4B). *Anaerobium* and *Geotalea* were most positively correlated to the LCFA% in substrate, followed by AD3 (phylum *Chloroflexi*) and *Paraclostridium* (Fig. 4C). Based on current knowledge, these four taxa have fermentative and acetogenic metabolism (Fincker et al., 2020; Hug et al., 2013; Kutsuna et al., 2019; Patil et al., 2015; Shelobolina et al., 2008). Similar to the trend obtained for LCFA%, the contour beta diversity plot with temperature regressed against the scores of the ordination (PCoA) showed that the three clusters remained distinctly separated despite being operated at different operational temperatures (Fig. 5A). The composition of microbes returned from

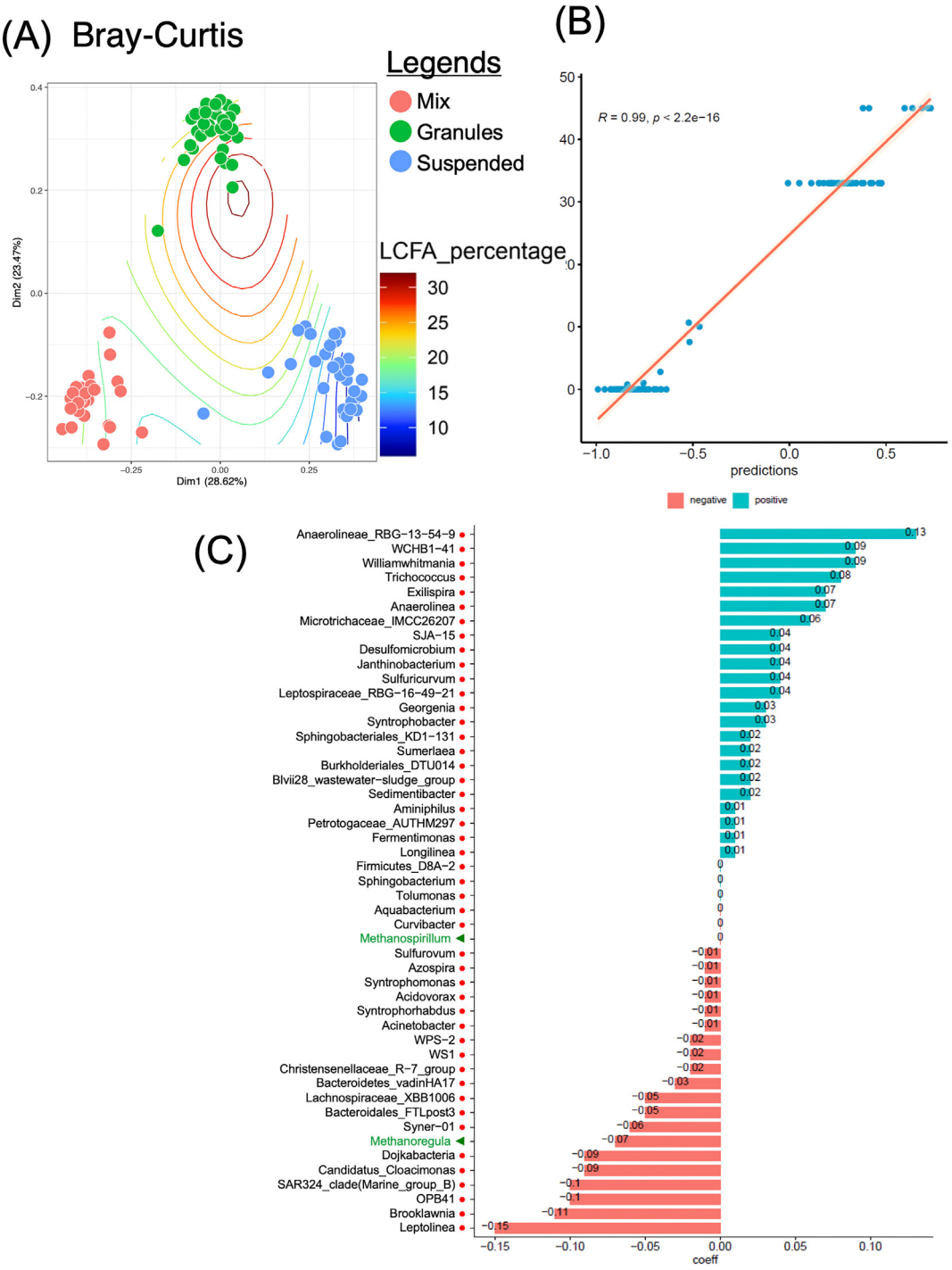

Fig. 4. a) Beta diversity contour plot with LCFA% regressed against the scores of the ordination (PCoA) using Generalised Additive Model (GAM); b) $\beta-$coefficients returned from CODA-LASSO procedure as two disjoint sets (those that are positively related, and those that are negatively related with the LCFA%) c) The predictions from the fitted model through CODA-LASSO was then further regressed against the true LCFA % to show agreement.

CODA-LASSO was able to segregate between the two temperature niches at 10 and 20 °C. Microbiomes at the two temperatures were clearly separated in terms of density plot of prediction returned from CODA-LASSO showing temperature-based delineation in the microbiomes (Fig. 5B). The archaeal taxa *Methanomethylovorans,* and bacterial taxa RBG–13–54–9, and p–1088–a5_gut_group were most positively correlated to temperature suggesting adaptability of these taxa to operational temperatures of 20 °C despite being sourced from mesophilic reactors. In AD reactors fed with oxytetracycline a temperature reduction from 35 °C to 15 °C increased the abundance of *Methanomethylovorans* (Yun et al., 2023), but their precise metabolic role in LtAD systems seeks further investigation. On the other hand, taxa from class *Phycisphaerae* - AKAU3564 sediment group, Pla1 lineage and SM23–30 were most negatively correlated to temperature suggesting their favorable growth at temperatures of 10 °C (Fig. 5C). AKAU3564 sediment group has often been found in marine sediments and psychrophilic anaerobic methane oxidizing consortia along with sulfate-reducing bacteria from class *Deltaproteobacteria* (Pernthaler et al., 2008; Trembath-Reichert et al., 2016; Yu et al., 2022), and despite a low abundance persisted as an anaerobic methanotrophic archaea in our LtAD reactors. Due to the lack of data on Pla1 lineage and SM23–30 and AKAU3564 sediment group in continuous LtAD reactors, it is not possible to state their role or suitability as an indicator organism for LtAD, but further work could explore functional relevance of this uncultured organism. Notably, no known syntrophic LCFA degraders were found to positively correlate to the LCFA % across the temperature ranges, indicating a role of novel taxa in anaerobic degradation of LCFA-rich substrates at 10–20 °C. Differences in microbial community based on OLR are presented in Supplementary Figs. 5A–C. Metadata regarding the LCFA degradation efficiency was not available in the original datasets and we recommend correlating microbial taxa active to LCFA degradation efficiency in LtAD systems in future studies. Moreover, performance of other reactor types, such as biofilm and membrane reactors should be evaluated for LtAD of LCFA-rich wastewaters. Hydrodynamics of an anaerobic system may promote temperature or concentration gradients (pH, substrate, and dissolved gases) that may impact the substrate availability to microbial consortia, dissolved gases stripping and the spatial distribution of microbial populations, and is related to their degree of dispersion, mixing, fluid pattern (Krsmanovic et al., 2021; Kundu et al., 2013; Lebranchu et al., 2017). LtAD for LCFA-rich wastewaters is still an emerging area of research for which the hydrodynamic aspects are important considerations for future research works.

Next, we applied a GLLVM model to analyze which microbial taxa (from the top 100 most abundant genera) are positively and negatively correlated with variables. The variables tested included the categorical variables – inoculum sources (VD, RD and Mix), inoculum type (granular and suspended), nucleic acid type (DNA), reactor configuration (DSC-FF, and EGSB), substrate type (LCFA-rich dairy, and distilled water), and the continuous (numerical) variables – LCFA %, OLR, and temperature. The results from GAM model showed similar trends to those obtained from GLLVM models. For example, the genera *uncultured Propionibacteriaceae*, *Longilinea*, *Christensenellaceae R7 group*, *Lactivibrio*, *candidatus Caldatribacterium, Aminicenantales*, *Syntrophus*, *Syntrophomonas*, *Smithella*, RBG-13-54-9, WCHB1–41, *Trichococcus*, *Proteiniclasticum*, SBR1031, *Lutibacter* and *Lentimicrobium* (Supplementary Fig. 6) are important to methanization of LCFA-rich wastewaters (Supplementary Fig. 6). *Tolumonas* was correlated to low temperatures of 10 °C and high LCFA% of 45 % (Supplementary Fig. 7). The contour beta diversity plots confirm that pivotal role of the *a priori* conditioning on the original microbial biomass for structuring the

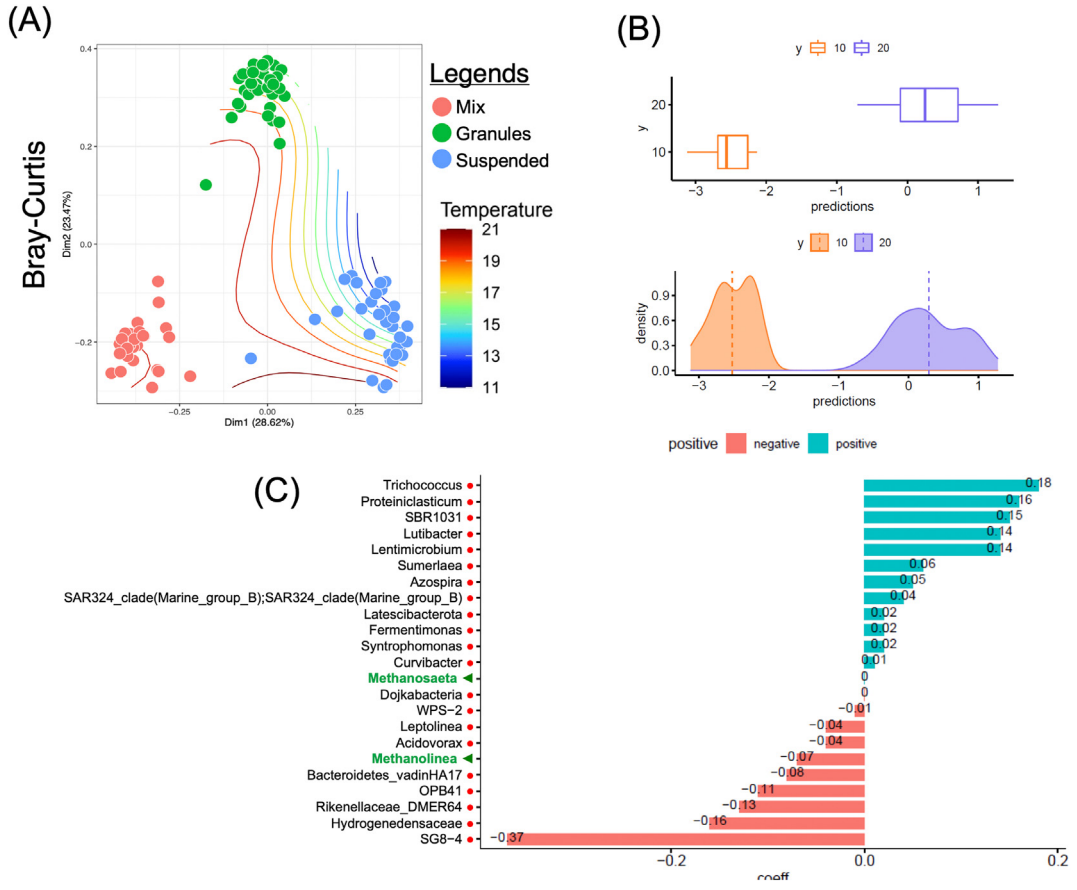

Fig. 5. a) Beta diversity contour plot with temperature regressed against the scores of the ordination (PCoA) using Generalised Additive Model (GAM); b) $\beta-$coefficients returned from CODA-LASSO procedure as two disjoint sets (those that are positively related, and those that are negatively related with the temperature) c) The density plot returned from the CODA-LASSO segregates the two temperature groups provides a graphical assessment of the classification accuracy (top: true; bottom: predicted from the procedure).

microbiome (Figs. 4A, 5A). This is counter to established theory which would suggest a more important role of the environmental variables, such as, LCFA concentrations or operational temperatures for driving community assembly and beta diversity.

3.5. Complexity stability relationship

Complexity-stability relationship of the clusters (suspended, mix, granular) when evaluated using the effective connectance (D^2) showed the clusters were distinct (Fig. 6A), and their dissimilarity versus overlap plots fitted well using different statistical measures (Jenson-Shannon divergences, Euclidean distances, and Spearman correlation values) (Fig. 6B–D). The number of species (n) in the clusters followed trend: mix > suspended > granules, ranging from 230 to 660 for cluster 'mix', 25–65 for cluster 'suspended' and 10–50 for cluster 'granules' (Fig. 6A). These trends remained same when using different statistical measures (Fig. 6A). Connectances for the clusters 'suspended' and 'granules' were similar and lower than of cluster 'mix' (Fig. 6A), representing redundancy in taxa for the clusters 'suspended' and 'granules'. High connectance in the cluster 'mix' represented an increased contribution of each taxon to the network, and removal of any of these taxa from the cluster 'mix' may destabilize the network. This holds validity since, the cluster 'mix' are cDNA samples and represent the active community in contrast to the total community represented in clusters granules and suspended. Within a microbiome, the active community is a subset of the total community that proliferates under the prevailing metabolic conditions. When operational conditions change, another redundant taxon may take over and proliferate to form the new active microbiome. Hence, the complexity-stability approach helped to assess the stability of clusters in anaerobic LCFA-degrading microbiomes from batch and continuous LtAD systems. A caveat of the stability-complexity relationship evaluation in current study is the lack of datasets from samples sequenced for both total DNA and cDNA, precluding a direct comparison between DNA and cDNA sequence effects and is recommended for future works. Future studies exploring stability of the microbial correlation and interaction networks may help in assessing the robustness of engineered microbiomes treating LCFA-rich wastewaters at low operational temperatures in order to select the optimal operational conditions.

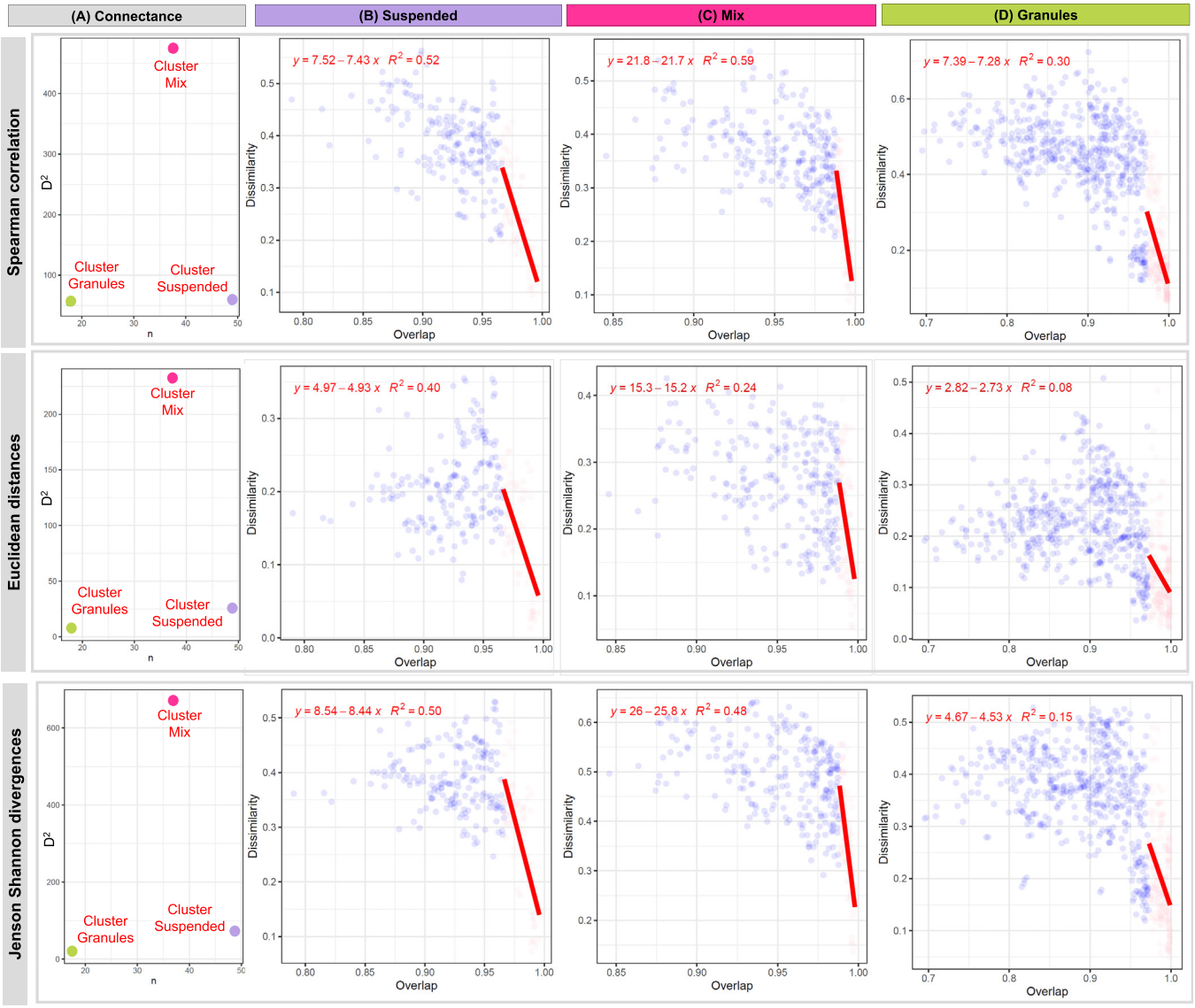

Fig. 6. Complexity-stability relationship of clusters (suspended, Mix, granular) where, (a) the effective connectance (D^2) is calculated based on fitting a linear regression to top 25 % overlap values for (b) cluster suspended, (c) cluster mix, (d) cluster granular using Jenson-Shannon divergences, Euclidean distances and Spearman correlation values.

4. Conclusions

Our comparative analysis showed inoculum characteristics (inoculum source, inoculum morphology) shaped the bacterial and archaeal diversity (species richness, species evenness) and beta-diversity patterns in anaerobic LCFA-fed microbiomes at 10–20 °C. Moreover, a priori effects associated to inoculum characteristics played a more prominent role than the LCFA percentage in substrate (0–45 %) or operational temperatures (10, 20 °C) in differentiating beta-diversity patterns in long-term reactor operation in batch mode (200 d) or continuous mode (150 d). These results suggest that the microbial community diversity of seed may continue to influence the microbial community diversity in long-term operation in open reactor systems (up to 150 d) as well as closed systems (up to 200 d). Core microbiomes of LtAD systems fed with LCFAs were found to be complex even at a very high prevalence (>95 %). The bacterial and archaeal genera represent the potential for acidogenesis, acetogenesis, β-oxidation and methanogenesis steps needed for anaerobic metabolism of LCFAs. Abundances of the psychrophilic bacteria RBG-13-54-9 from family *Anaerolineae*, and taxa WCHB1–41 and *Williamwhitmania* were correlated to high LCFA% and may represent novel LCFA-degrading taxa in LtAD systems. Many taxa found in the core microbiomes, heat trees and associated with high LCFA% and low temperatures, were uncultured or have candidatus status, highlighting the high abundance of microbial dark matter in LtAD systems treating LCFAs at temperatures of 10–20 °C. The role of these uncultured taxa needs additional investigation. Complexity-stability analysis showed that 'granular' and 'suspended' clusters had higher stability, in comparison to the cluster 'mix'. Future work exploring the stability of the LCFA-degrading microbiomes under more expansive test conditions at low ambient temperatures would be useful to select optimal operational conditions further in view of the *a priori* effects of inoculum.

CRediT authorship contribution statement

Suniti Singh: Conceptualization, Data curation, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Ciara Keating:** Data curation, Methodology, Formal analysis, Writing – review & editing. **Umer Zeeshan Ijaz:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Supervision. **Francis Hassard:** Conceptualization, Writing – review & editing, Supervision.

Data availability

The data is publicly available.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.162420.

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