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Resource recovery from the anaerobic digestion of food waste is underpinned by cross-kingdom microbial activities

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ABSTRACT

As the human population grows on the planet so does the generation of waste and particularly that of food waste. In order to tackle the world sustainability crisis, efforts to recover products from waste are critical. Here, we anaerobically recovered volatile fatty acids (VFAs) from food waste and analysed the microbial populations underpinning the process. An increased contribution of fungi relative to bacteria was observed throughout the reactor operation, with both kingdoms implicated into the main three steps of anaerobic digestion occurring within our systems: hydrolysis, acidogenesis and acetogenesis. Overall, *Ascomycota*, *Proteobacteria* and *Firmicutes* were found to drive the anaerobic digestion of food waste, with butyrate as the most abundant VFA likely produced by *Clostridium* using lactate as a precursor. Taken together we demonstrate that the generation of products of added-value from food waste results from cross-kingdoms microbial activities implicating fungi and bacteria.

1. Introduction

Microorganisms underpin the functioning of our ecosystems which in turn provide a multitude of services on which we depend, including climate regulation, nutrient cycling, crop productivity and human health (Baveye et al., 2016). Additional vital services are provided through for example harnessing microbial processes to decompose various anthropogenic wastes (Graham and Smith, 2004), at a rate and efficiency unmatched by abiotic methods. Anaerobic digestion (AD) is one such engineered system where organic wastes are rapidly degraded while producing methane that can be used as a source of biofuel (Gujer and Zehnder, 1983). AD is a four-step microbial sequential process encompassing hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The source of feedstock is a pivotal factor determining the feasibility of AD (Gebrezgabher et al., 2010), where typically a continuously available source with sufficient organic matter is required. While crops can be grown to provide AD biomass, the use of organic wastes instead represents a more sustainable alternative that fits into the circular bio-economy model (Venkata Mohan et al., 2016).

Food waste represents a significant portion of anthropogenically derived organic waste and initial broad estimates by the FAO (2011) suggest that one third of food produced for human consumption goes to waste. More recent data estimate that 14% of food goes to waste during production processes alone (FAO, 2019). While post-consumer wastage can be vastly minimised, those from production (peelings, damaged or diseased matter, inedible plant parts) are likely to remain at similar, or increasing, values as populations grow. This food supply chain waste represents a high-value feedstock for AD (Matharu et al., 2016). Thus, an opportunity exists to shift food waste from a pollutant to a renewable resource, consequently lowering waste-related challenges in the long term (Morales-Polo et al., 2018).

Conventionally, the desired product of the AD process was biogas as a source of methane; however, research is now focusing to the generation of intermediate compounds (Nzeteu et al., 2018; Slezak et al., 2020), in the manner of biorefining. AD intermediates such as VFAs can be used in the production of food additives, pharmaceuticals, fungicides, lubricants and paints, amongst other applications (Wainaina et al., 2019). AD generated VFAs hence represent renewable alternatives to the VFAs, which are currently produced via petrochemistry (Baumann and Westermann, 2016). Rate limiting steps however, are frequently encountered when using complex substrates such as food waste for AD feedstocks (Ma et al., 2013). Food waste typically has a significant

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portion of complex carbohydrates including lignocellulosic and/or hemicellulosic compounds, (25-30% of total solids; (Fisgativa et al., 2016; Nzeteu et al., 2018)) originating from plant matter, which are challenging to hydrolyse (Ma et al., 2013; Tomei et al., 2009; Zhang et al., 2014). In an effort to overcome this, various methods have been tested including alkaline (Cheah et al., 2019; Fang et al., 2019), thermal (Zhang et al., 2019), acid (Cheah et al., 2019) and enzymatic pretreatments (Kim et al., 2006). These approaches however tend to increase operating costs and render the process economically unattractive. Hence, biological strategies whereby operational conditions are tailored to promote the growth and persistence of key microbial hydrolysers within AD reactors represent promising alternatives. In that context, leach-bed reactor (LBR) configuration has shown encouraging result in term of efficient hydrolysis and VFA production from food waste (Nzeteu et al., 2018; Hussain et al., 2017; Xiong et al., 2019). This could be partly attributed to the implementation of leachate recirculation to dilute inhibitory compounds as well as increase moisture in the solid bed thus facilitating microbial growth and activity while reducing the overall water requirement (Nzeteu et al., 2018; Hussain et al., 2017; Xiong et al., 2019). In order to more successfully implement biological strategies, however, knowledge of key AD microbial processes is essential. This includes insights into which consortia drive the four phases of AD, thereby allowing for targeted interventions to overcome the identified rate limiting steps. This knowledge can then underpin process optimisation through alteration of operating parameters in order to suit the lifestyle strategies of the microbial group(s) implicated (Kreuzinger et al., 2004; Vuono et al., 2015; Ziganshin et al., 2016). To date, much research into AD microbiomes has involved taxonomic characterisation, principally undertaken using 16S rRNA profiling thus confining findings to bacteria and archaea only. Metaproteomics is perhaps the most informative in terms of assessing microbial processes occurring in-situ (Joyce et al., 2018; Heyer et al., 2019; Siggins et al., 2012) and allows for the identification of proteins from all kingdoms of life.

The aim of this work was therefore to investigate the microorganisms present in replicated AD reactors treating food waste, with process parameters optimised for organic acid production. To this end, we used DNA and RNA for 16S rRNA profiling and metaproteomics to investigate the microorganisms involved in the different steps of substrate breakdown.

2. Materials and methods

2.1. Reactor operation

Three replicate leach-bed reactors (R1, R2 and R3) with a total and working volume of 6 L and 3 L, were operated in a semi-continuous mode at 37 °C with 14-day SRT (solid retention time) as described previously (Nzeteu et al., 2018). For the first batch, 1 L of water was added to the lower compartment, prefilled with pumice stone, while the upper compartment was loaded with 80 g VS L^{-1} of food waste (FW) (VS: volatile solids) (Nzeteu et al., 2018), 40 g of sodium bicarbonate ($NaHCO_3$) and inoculated with granular sludge from a full-scale mesophilic reactor (Carbery Milk Products, Ireland) at the VS ratio of 0.25 (inoculum/FW). The subsequent batches were inoculated with digestate and leachate from the previous batch at a VS ratio of 0.25. Leachate recirculation was set for 1 h day^{-1} at 20 mL min^{-1}. Seven batches were run over 97 days of operation. Batch 7 was chosen for in-depth investigation after demonstrating stable and reproducible reactor performance with regards to VS removal, VFA production and soluble chemical oxygen demand (sCOD) profile across 4 consecutive batches (Nzeteu et al., 2018). Duplicate 2 mL leachate samples were collected on day 0, 1, 3, 7 and 14 and filtered through a 0.2 μm filter. Filtrates were used for pH, ammonia (NH_3), sCOD, and VFA analysis. For microbiome analysis, duplicate samples of 7 g digestate and 40 mL leachate were withdrawn from each reactor on day 0, 1, 3 and 7 to provide insights into hydrolysis (day 0, 1 and 3) and fermentation processes (3 and 7) as indicated by sCOD and VFA analysis. Physical and chemical characteristics of the food waste are described in Nzeteu et al. (2018).

2.2. Chemical analysis

Leachate samples were analysed for sCOD concentrations according to the Standing Committee of Analysts Methods (1985). Total solids (TS) and VS from food waste and digestate samples were determined using standard methodologies (APHA, 2005). Ammonia concentrations in leachate were measured using HACH methods and test kits (HACK Odyssey). Biogas methane content and VFAs (C2-C6) were quantified as previously described (Nzeteu et al., 2018). Lactic acid and ethanol assay kits (Megazyme) were used to measure lactic acid and ethanol concentrations.

2.3. Microbiome recovery and biomolecule co-extraction

Microbial cells were recovered from leachate and digestate samples as described in Thorn et al. (2019) and immediately resuspended in 2 mL RNAlater® solution (Ambion™) before incubation at room temperature for 3 h. RNA later suspensions were transferred into sterilised 2 ml tubes and centrifuged at 8000 ×*g* for 15 min. Cell pellets were snapped frozen in liquid nitrogen and stored at −80 °C. DNA, RNA and proteins were co-extracted from 0.2 g of leachate and 0.6 g of digestate microbial cells pellets at each time points using NucleoSpin TriPrep kits (Macherey-Nagel). RNA samples were DNase treated with TURBO DNA-free™ Kit (Ambion by Life Technology). Control PCRs using DNase treated products as templates (neat, 1/5 and 1/10 dilutions) and primer pair 515F/806R (Caporaso et al., 2011) were carried out in 25 μL reaction containing: 0.2 μM of each primer, 200 μM of each deoxynucleoside triphosphate (dNTPs), 1.5 mM magnesium chloride ($MgCl_2$), 1× ammonium and 5×10^{-2} U/μL of Taq polymerase (Bioline). PCR conditions consisted of 94 °C for 30s, 30 cycles at 50 °C for 30s and 72 °C for 30s, and a final step at 72 °C for 2 min. PCR products were visualised on a 1% agarose gel containing 5×10^{-4}mg mL^{-1} ethidium bromide. Once RNA samples were confirmed to be DNA free, complementary deoxyribonucleic acid (cDNA) was generated using the superscript reverse transcriptase III (Invitrogen).

2.4. 16S rRNA sequencing and bioinformatic analysis

V4 region of 16S rRNA from DNA and cDNA samples was amplified in triplicate using 515F and 806R primers in 25 μL reactions as follows: 1× Q5® Reaction Buffer, 200 μM of each dNTPs, 0.2 μM of each primer and 2×10^{-2}U μL^{-1} Q5 High-Fidelity DNA Polymerase (New England BioLabsinc). PCR conditions were the same as above. Triplicate PCR products for each sample were combined into a single volume and purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). Purified amplicon concentrations were determined (Qubit dsDNA HS assay kit; Invitrogen) and normalized to 20 ng μL^{-1} before pooling. Samples underwent paired-end sequencing on MiSeq Illumina platform (Research and testing laboratory (RTL), Texas US). 94 samples (48 DNA and 46 cDNA samples) were analysed (duplicate samples from digestate and leachate from the triplicate reactors on day 0, 1, 3 and 7). RNA yield obtained from the digestate fraction of R1 on day 0 was below detection limit and thus was not included. Sequencing data were analysed using the RTL methodology (RTL, 2019). Sequences are deposited in NCBI's Sequence Read Archive under accession number PRJNA692345.

2.5. Metaproteomics

Proteins from digestate samples collected on day 1, 3 and 7 were analysed. Protein concentrations were determined using the Non-Interfering Protein Assay (Calbiochem). Samples were normalized (24 μg) and run on a 12% SDS-PAGE gel, as outlined previously (Joyce et al.,

2018). In-gel tryptic digestion was conducted prior to nanoflow liquid chromatography-electrospray ionisation tandem mass spectrometry analysis (BSRC Mass Spectrometry and Proteomics Facility, University of St Andrews, UK) as described by Thorn et al. (2019). MetaProteomeAnalyser (MPA) software (Muth et al., 2015) was used for metaprotein assignment. Database searches were run using the X! Tandem (version ALANINE; 2017.02.01) and OMSSA (version 2.1.8) algorithms, and spectra identified against UniProt/SwissProt (release 2016_10) database. Search parameters were as described by Thorn et al. (2019). Target decoy searching was systematically performed and protein hits grouped into metaproteins, if they had at least one peptide in common, with a false discovery rate of <1% and using the lowest common ancestor for phylogenetic assignment. Protein annotation was achieved using NCBI, UniProt and KEGG (Muth et al. (2015). As 82% of fungal proteins were of unknown COG category, all proteins were searched against KEGG Orthology (KO) database. Where a COG entry was absent, a KO entry was used if possible. This increased the number of fungal orthologous groups identified from 18% to 76% (Table S1). Data visualization was performed using Chordomics (McDonnell et al., 2019). Interactive versions of the plots are available here: https://cethorn.shinyapps.io/nzeteuetalchordomics/. Finally, proteins were grouped into those associated with the key stages of anaerobic digestion (Sikora et al., 2019). The proteomics data can be accessed at https://doi.org/10.17630/e2603185-dc54-4034-9c73-bb20749caf60.

2.6. Statistical analyses

All analyses were performed in R (R Core Team (2017)) using the vegan package (Oksanen et al., 2013). Bray Curtis dissimilarity index was used to determine the distance between 16S rRNA samples and was ordinated using 2D nMDS (stress <0.2; (Clarke, 1993)). Grouping of samples as a function of days or reactor fraction (leachate or digestate) was tested for statistical significance using analysis of similarity (ANOSIM). Percentage contribution of each genus to the grouping was calculated using SIMPER analysis and Kruskal–Wallis tests were performed to assess whether the relative abundance of each genus contributing to the grouping was significantly different over time. To identify relationships between VFA production and dominant microbial genera (identified by SIMPER analysis) in all three datasets (DNA, RNA and protein), data were first tested for normality with the Shapiro Wilk Test. As some variables were non-normally distributed, Spearman's non-parametric correlations were performed between VFAs and each of the microbial datasets, where p values were corrected for multiple testing (FDR adjustment). As proteins were analysed on days 1, 3 and 7, data from these days only were used for correlations.

3. Results

3.1. Rapid food waste hydrolysis occurred within the first three days of reactor operation

A rapid increase in sCOD in the reactor leachates was observed within the initial three days of operation, indicative of food waste hydrolysis (Fig. 1A). This was accompanied by a sharp decrease in pH from 7 to 5.2 within the same timeframe, after which the leachate pH increased to reach 6, 6.5 and 7.2 in R1, R2 and R3 on day 13 (Fig. 1B). Ammonia concentrations, indicative of protein hydrolysis, increased in all reactors from day 1 to 3 followed by a plateau and a further increase from day 7 to 13 (Fig. 1C). Overall, leachate ammonia concentrations ranged from 8 g L^{-1} to 10 g L^{-1} with highest concentrations reaching 14 g L^{-1} in R1. By the end of the trial, 64.8 ± 4.3% VS removal was achieved in the reactors. On day 13 (when reactors were drained), leachate VFA concentrations reached 77.57, 74.04, and 64.12 g sCOD L^{-1} in R1, R2 and R3 (Fig. 1D). Biogas methane content accumulated in R1, R2 and R3 was 0.1, 3.7 and 0.2%. VFA production occurred between days 3 and 13 (Fig. 1D) and butyric acid was the most abundant VFA accumulated (ranging from 24.9 to 32.9 g COD L^{-1}) followed by acetic acid (from 10.1 to 13.7 g COD L^{-1}), propionic acid (from 6.4 to 16.9 g COD L^{-1})

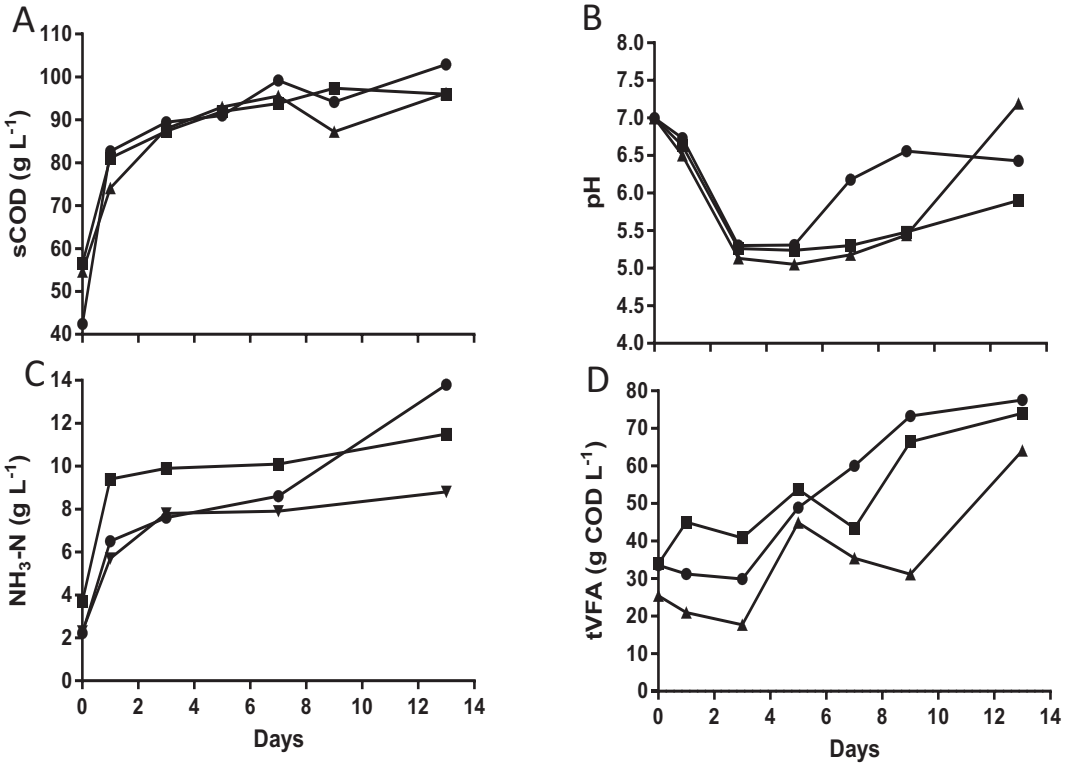

Fig. 1. Profile of: A) soluble chemical oxygen demand (sCOD); B) pH; C) ammonia nitrogen (NH_3-N) and D) volatile fatty acid (VFA) in leachate samples from R1, R2 and R3 throughout the reactor run. R1, R2 and R3 are the biological replicate reactors.

and caproic acid (from 7.2 to 12.2 g COD L^{-1}) (Fig. 2). Lactic acid concentrations increased from day 0 to 3 and subsequently decreased from day 5 to 13 (Fig. 2), while ethanol concentrations decreased from day 0 to 1 and did not change until day 13 (Fig. 2).

3.2. Temporal shift in microbial taxa during food waste degradation

DNA and cDNA profiling from leachate and digestate samples on day 0, 1, 3 and 7 led to the identification of 3368 species-level OTUs (96% sequence similarity; Fig. S1A and B). Bray Curtis dissimilarity index analysis revealed clear sample clustering as a function of time and as a function of reactor fractions (leachate and digestate) (Fig. 3; Table S2). Microbial communities associated with the observed clusters were more similar ($p < 0.01$) within samples collected on the same day than between samples collected on different days at both DNA (R value of 0.66) and cDNA (R value of 0.77) level (Fig. 3). Leachate and digestate technical replicates shared 70 to 95% similarity with the highest community dissimilarity observed for digestate samples likely reflecting the complex nature of this matrix (Fig. S2). For DNA samples, *Enterococcus*, *Clostridium*, *Lactobacillus*, *Bifidobacterium* and unclassified *Bifidobacteriaceae* accounted cumulatively for up to 83% of community differences across sampling days (Table S3). The same microbial taxa (*Enterococcus*, *Clostridium*, *Lactobacillus*, *Bifidobacterium* and unclassified *Bifidobacteriaceae*) contributed to the majority (73%–80%) of the dissimilarity between cDNA samples, in addition to *Corynebacterium*. All these influential genera were found to be differentially abundant ($p < 0.05$) as a function of time in both nucleic acid fractions (Fig. S3), with the exception of *Clostridium* in DNA samples. The relative abundance of *Enterococcus* saw a notable increase between day 0 and 1 of approximately 5 fold in the DNA dataset (Fig. S3A) and 3.5 fold in the cDNA dataset (Fig. S3B). This coincided with a reduction in *Bifidobacterium* relative abundance, which by day 3 had increased back up to similar levels (DNA) or slightly in excess (cDNA) of their original abundance on day 0 (Fig. S3). *Lactobacillus* relative abundance increased on day 3 and fell again by day 7. Similar trends were seen in DNA and cDNA datasets, aside from the added contribution of *Corynebacterium* and *Clostridium* to the cDNA (Table S3). Taken together, these results suggest that *Enterococcus* were involved in the early stages of food waste degradation, while *Bifidobacterium*, *Lactobacillus* and *Clostridium* were involved in the latter stages. Archaea represented less than 1% of the total microbiota in all samples and were likely inhibited by the low pH initially prevailing in the reactors. This observation is in agreement with the low level of methane production recorded during the reactor run.

3.3. Fungal to bacterial protein ratio increases throughout the reactor trial

Protein profiling of the 9 samples analysed (digestate from R1, R2 and R3 on day 1, 3 and 7) led to the identification of 2884 metaproteins (Tables S4 and S5). These were predominantly assigned to bacteria (78%), with 15% assigned to fungi, 2% to archaea and 5% of unknown origin (Fig. 4A). Overall protein assignment for prokaryotes was in good agreement with that detected by 16S rRNA profiling (Fig. S4). The number of bacterial proteins decreased over time, while those assigned to fungi increased as the reactor run progressed. On day 1 fungal proteins represented 6.4% of the total proteins, increasing to 11% by day 3 and to 32% by day 7 (Fig. 4A). The number of archaeal proteins remained low throughout the trial, representing less than 2% of the total proteins. This observation is in accordance with 16S rRNA profiling, and demonstrates the successful negative selection against methanogens in our reactors. The majority of fungal proteins belonged to the phylum *Ascomycota* (94%) while bacterial proteins were principally expressed by *Firmicutes* (55%), *Proteobacteria* (23%) and *Actinobacteria* (10%) (Fig. 4B). More than a third (39%) of *Ascomycota* proteins were assigned to *Saccharomycetales*. However, their relative abundance decreased throughout the reactor run (https://cethorn.shinyapps.io/nzeteuetalchordomics/). Conversely, there was a steady increase in *Sordariomycetes* proteins, rising from 6.8% (day 1) to 13% (day 3) and finally to 30% (day 7) with the majority assigned to *Neurospora crassa*. The remaining fungal proteins were expressed by *Schizosaccharomycetales* and *Eurotiomycetes* (https://cethorn.shinyapps.io/nzeteuetalchordomics/). In contrast to fungi, the proportions of phyla accounting for the majority of bacterial metaproteins (*Firmicutes*, *Proteobacteria* and *Actinobacteria*) remained relatively stable over the three sampling days (https://cethorn.shinyapps.io/nzeteuetalchordomics/). Proteins assigned to Firmicutes were predominantly from *Lactobacillales* (71%), *Bacilli* (12%) and *Clostridia* (10%). *Proteobacteria* proteins were mostly assigned to *Enterobacteriales*, while 95% of *Actinobacteria* proteins were expressed by *Actinomycetales* and *Bifidobacteria* (https://cethorn.shinyapps.io/nzeteuetalchordomics/).

3.4. Cross-kingdom microbial activities underpins food waste AD

Protein hits were grouped into their associated functional category, using a custom unified orthology (COG and KEGG) (Fig. S5; Fig. 4B; https://cethorn.shinyapps.io/nzeteuetalchordomics/). The most abundant functional groups were translation, ribosome structure and biogenesis followed by carbohydrate metabolism and cellular processes. Most

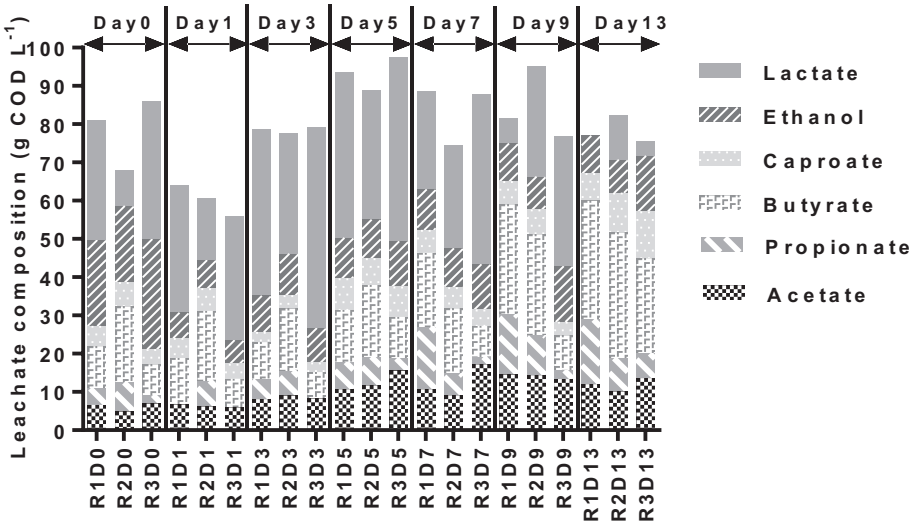

Fig. 2. Profile of carboxylate and ethanol in R1, R2 and R3 leachate samples throughout the reactor run. Value at each time point is the average of duplicate measurements. R1, R2 and R3 are the biological replicate reactors. D stands for day.

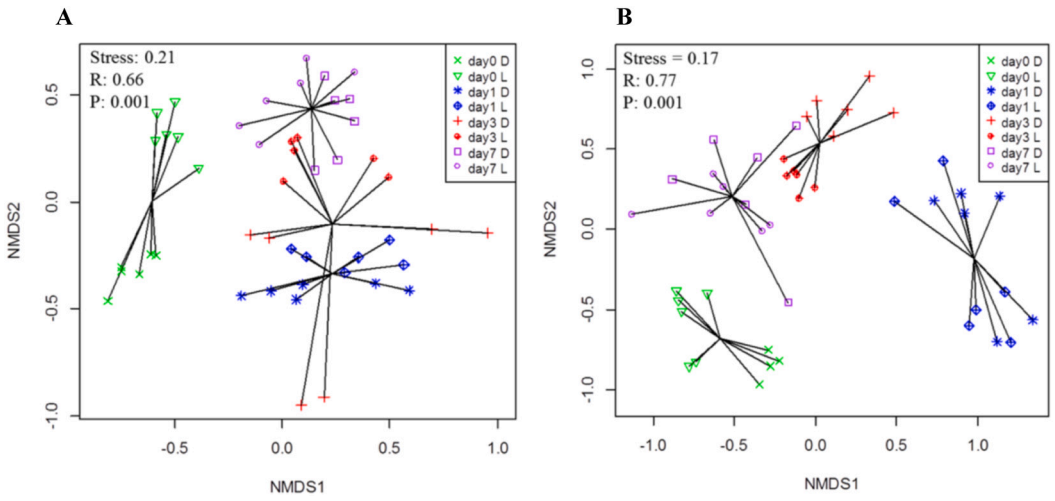

Fig. 3. nMDS plot illustrating microbial community shifts analysed from A) DNA and B) cDNA based 16S rRNA Miseq sequencing. The community profile on the plot at each sampling point is represented by a specific colour (green for day 0, blue for day 1, red for day 3 and purple for day 7), while the community present in each fraction (leachate = L and digestate = D) of the reactors is illustrated by symbols. Cluster significance: A) $R = 0.66$ and $p = 0.001$; B) $R = 0.77$ and $p = 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

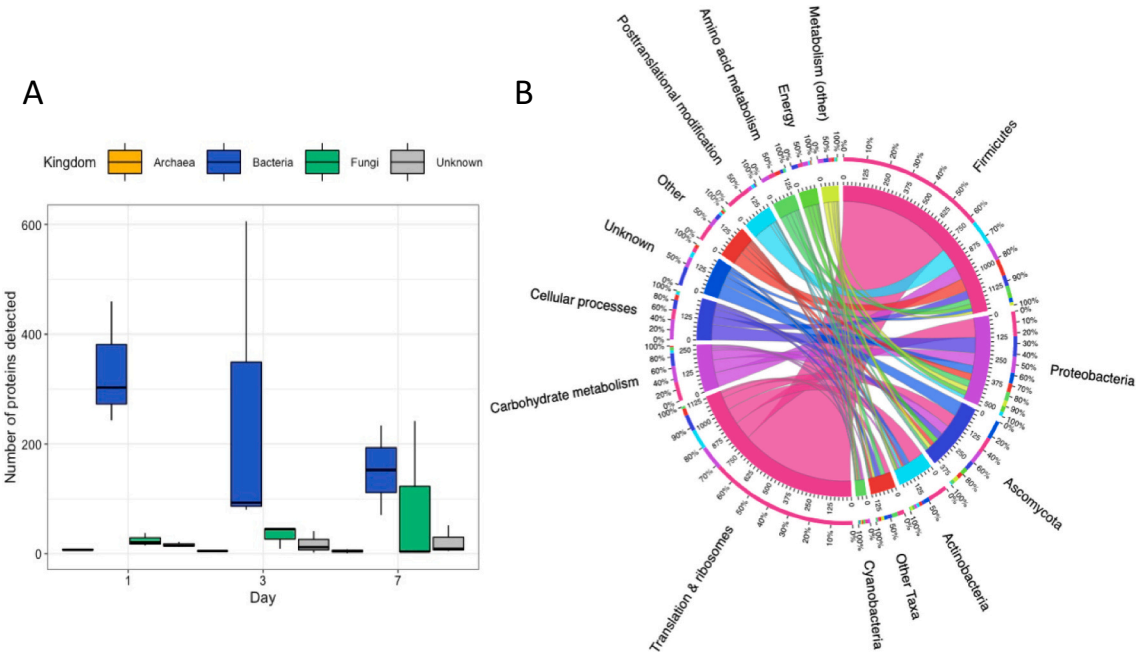

Fig. 4. Overview of protein assignment and function. A) Number of proteins assigned to archaea, bacteria and fungi on each sampling day (day 1, 3 and 7). Proteins assigned to category 'Unknown' corresponds to protein with peptide hits found across all kingdoms. B) Chord plot representing all proteins identified throughout the reactor run (sum of each sampling day) with protein assignment at phylum level on the right and protein function on the left. Interactive view of the plot: https://cethorn.shinyapps.io/nzeteuetalchordomics/.

proteins associated with carbohydrate metabolism were assigned to *Lactobacillales* (25%), *Enterobacteriales* (20%) and *Saccharomycetales* (11%) (https://cethorn.shinyapps.io/nzeteuetalchordomics/). The number of proteins involved in translation, ribosome structure and biogenesis and cellular processes decreased throughout the reactor run, while the number of those involved in energy production and conversion, mostly assigned to *Lactobacillales and Sordariales*, increased. Despite the relatively high fat content of the food waste (28%; (Nzeteu et al., 2018)), only few proteins (n = 34) were assigned to the category of lipid metabolism, and those were primarily expressed by *Proteobacteria* (https://cethorn.shinyapps.io/nzeteuetalchordomics/). Hydrolytic proteins were mostly assigned to *Ascomycota,* and *Firmicutes*, with the largest contribution from Proteobacteria (72% of which were *Enterobacteriales* of diverse species) (Fig. 5; https://cethorn.shinyapps.io/nzeteuetalchordomics/). All hydrolytic enzymes assigned to this clade were beta-glucosidase/beta-glucuronidase, responsible for cleaving galactose from carbohydrates. Contrastingly, *Ascomycota* were found to express a diverse array of hydrolytic enzymes, including beta-fructofuranosidases, lysophospholipid hydrolases and various sugar hydrolases (https://cethorn.shinyapps.io/nzeteuetalchordomics/). The number of acidogenic proteins decreased with time, and were dominated, in all samples, by those assigned to *Firmicutes,* of which *Lactobacillales* contributed the most (84% of acidogenic proteins), followed by *Bacillales* and *Clostridiales* (Fig. 5). These included a number of proteins involved in

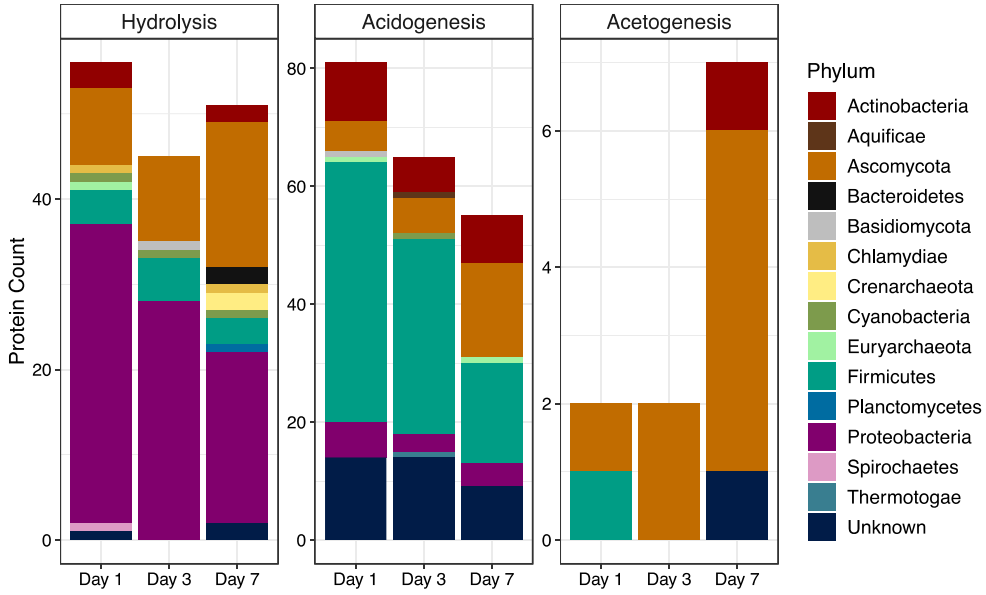

Fig. 5. Number of proteins assigned to the 3 steps of the anaerobic digestion process taking place within the reactors: hydrolysis, acidogenesis and acetogenesis. Protein numbers and phylogenetic assignments at the phylum level are displayed for each sampling day (day 1, 3 and 7).

glycolysis and in VFA production notably lactate dehydrogenase (pyruvate to lactate) primarily assigned to *Enterococcus* and *Lactobacillus* and butyryl-CoA dehydrogenase, involved in butyrate production, from *Clostridium acetobutylicum*. In addition, a total of four 3-hydroxybutyryl-CoA epimerases (three assigned to *Pseudomonas* and one to *Escherichia*), also involved in butyrate production, were detected on the three sampling days (https://cethorn.shinyapps.io/nzeteuetalchordomics/). Acetogenic proteins, mostly assigned to *Ascomycota*, increased over time, and were in low numbers (n = 11) with the majority detected on day 7 (n = 7; Fig. 5). In addition, five acetate kinase (acetyl-P to acetate) assigned to diverse bacterial species were detected. However, due to their role in both acetate and propionate production these were included under acidogenesis. Finally, no proteins associated with methanogenesis were identified in the entire protein dataset (using EC numbers associated with either hydrogenotrophic or acetoclastic methanogenesis).

3.5. *Clostridium* is involved in butyric and caproic acid production

Correlations between VFAs and four key genera (*Enterococcus*, *Lactobacillus*, *Bifidobacterium,* and *Clostridium*) across the three datasets were analysed (Table 1). *Enterococcus* showed a strong negative correlation with acetic acid (in both DNA and cDNA data; Table 1) with a relative abundance declining rapidly from day 1 to day 3, while acetate began to accumulate in the reactors (Fig. 2). In contrast, acetate concentration correlated positively with relative abundances of *Bifidobacterium* in both DNA (R = 0.84) and cDNA (R = 0.74) datasets (Table 1). While the highest number of proteins assigned to *Bifidobacterium* were detected on day 3, increasing from 16 proteins on day 1 up to 41 proteins on day 3 (and falling again to 18 on day 7), no statistically significant correlations were seen between *Bifidobacterium* protein counts and acetate production. A negative relationship was seen between *Clostridium* and lactate across all three datasets (DNA, cDNA and protein). Unique to the cDNA fraction, was a positive correlation between *Bifidobacterium* and lactic acid (R = 0.52). Similarly, a negative correlation between *Lactobacillus* and lactate was only seen in the protein datasets, and when looking at the raw data, this counter-intuitive relationship could be explained by the lower lactate concentration in R2, which conversely saw the highest protein count assigned to *Lactobacillus*. Butyric acid was positively associated with *Clostridium* in both 16S rRNA datasets (R above 0.6), as was caproic acid (R above 0.66). Caproic acid was also negatively correlated to *Lactobacillus* relative abundances in the cDNA dataset, and while a similar correlation was seen in the DNA dataset, it was not statistically significant.

Table 1
Spearman's correlation coefficients of the 4 influential genera (identified from 16S rRNA dataset) against acetate, butyrate, caproate and lactate. For DNA and RNA (cDNA) datasets, relative abundances of each genus were used, while for protein dataset the proteins counts per genus was used. Only correlations with $p < 0.05$ (FDR adjusted) are shown.

		Acetate	Lactate	Butyrate	Caproate
DNA	*Bifidobacterium*	0.84			
	Clostridium		−0.67	0.78	0.78
	Enterococcus	−0.83			
	Lactobacillus				
cDNA	*Bifidobacterium*	0.74	0.52		
	Clostridium		−0.48	0.60	0.66
	Enterococcus	−0.78			
	Lactobacillus				−0.48
Protein	*Bifidobacterium*				
	Clostridium		−0.82		
	Enterococcus				
	Lactobacillus		−0.68		

4. Discussion

4.1. Rapid hydrolysis and acidogenesis promote VFA accumulation and methanogenesis inhibition

Food waste solubilisation occurred during the initial three days of reactor run, as indicated by the rapid increased in sCOD, and decrease in pH within that period (Fig. 1A and B). Furthermore, increases in ammonia nitrogen concentrations detected in leachate provided evidence of protein hydrolysis (Fig. 1C) (Lukitawesa et al., 2020; Selvam et al., 2010; Wang et al., 2014; Yin et al., 2016). The produced ammonia nitrogen likely buffered the system as indicated by the increase in pH of the leachate towards the end of the trial (Fig. 1B). A similar pH pattern was reported by Lay et al. (2003) during the breakdown of protein-rich wastes, where over the initial 2 days the pH dropped from 7 to 5.8 after which it increased to 6.5, a pattern which was not seen when carbohydrate rich wastes were used. To promote VFA accumulation, the AD process must be halted at the acidification step, which can be done by

increasing substrate loading rates, amongst other means (Lukitawesa et al., 2020; Wainaina et al., 2019). We used a high loading rate (80 g VS FW L^{-1}) to shock-load the system, which promoted VFA accumulation while lowering the pH and inhibiting methanogens. Furthermore, the use of digestate and leachate from previous batches to inoculate new batches (semi-continuous operation), likely favoured the enrichment of acidogens. As a result, high VFA production was recorded, reaching 40.9 ± 2.77 g sCOD kg^{-1} FW, and exceeding rates reported in the literature under similar operating conditions and reactor configuration (Yesil et al., 2014; Slezak et al., 2020). Even though Xiong et al. (2019) reported a lower VFA concentration of 24.3 g COD L^{-1} from a similar set up, their VFA yield was higher than achieved herein (600.9 g COD kg^{-1} VS versus 170.6 (±2.77) g COD kg^{-1} VS). This difference may be a consequence of maintaining pH at 6 throughout the reactor run (Xiong et al., 2019). Similarly, Hussain et al. (2017) achieved slightly higher sCOD accumulation (530 g COD kg^{-1} VS versus 417 g COD kg^{-1} VS) and VFA yield (247 g COD kg^{-1} VS versus 170 g COD kg^{-1} VS) likely resulting from their high operating temperature (50 °C) and constant pH (7). Together this demonstrates the importance of high OLRs and pH control for achieving VFA accumulation while digesting food waste. We report butyric acid as the most abundant VFA produced likely using lactate as a precursor (Fig. 2, Table 1) (Nzeteu et al., 2018; Zhu et al., 2015; Zhu et al., 2017). While Doğan and Demirer (2009) and Yesil et al. (2014) recorded acetic acid as the most abundant VFA, Xiong et al. (2019) and Slezak et al. (2020), like us, reported butyric acid as the major VFA produced in their systems. Increased butyric acid production is of particular importance due to its high commercial value as a precursor for butanol production (Lee et al., 2014; Richter et al., 2012).

4.2. *Ascomycota, Proteobacteria and Firmicutes drive food waste AD*

Our microbial datasets were dominated by a limited number of phyla, which were also identified as dominant during an in-depth characterisation of various food wastes (Fisgativa et al., 2017) - *Ascomycota, Proteobacteria* and *Firmicutes* - perhaps indicating the role of the initial feedstock microbial load in the digestion process. *Firmicutes* are typically one of the most abundant phyla in food waste AD systems (Bengelsdorf et al., 2013; Li et al., 2015; Li et al., 2018), and this was confirmed by both our nucleic acid and protein datasets. *Lactobacilli* contributed the largest proportion of proteins within this phyla, in agreement with Moestedt et al. (2019). This might be attributed to their diverse fermentative capabilities, underpinning their wide industrial use for the production of various fermented foods. Additionally, as lactic acid bacteria (LAB) are unable to produce most amino acids, they scavenge them from their surroundings, typically by breaking down proteins and peptides (König and Fröhlich, 2017). Food waste therefore, with its high protein content represents an ideal substrate for these species. In contrast to the aforementioned studies, *Bacteroidetes* were barely detected in our proteomic dataset, and were of low relative abundance (< 5%) in the nucleic acid data. Gut microbiota research has revealed this phylum to be acid-intolerant (Ilhan et al., 2017; Walker et al., 2005), thus perhaps the low *Bacteroidetes* numbers herein result from the acidic conditions prevailing in our reactors.

While, fungal characterisation of anaerobic digesters is sparse relative to their bacterial counterparts, the presence of *Saccharomycetales* as a dominant fungal group in AD systems has been noted in previous research (Bengelsdorf et al., 2013; Bücker et al., 2020; Kazda et al., 2014; Sun et al., 2015; Young et al., 2018) in addition to this research. Many members of this clade are able to grow anaerobically by fermenting sugars to acetaldehyde, which is then converted principally to ethanol, but also to acetate (Simpson-Lavy and Kupiec, 2019). Additionally, *Saccharomyces* have been shown to remove around 50% of both protein and lipid content of food waste under fermentative conditions (Suwannarat and Ritchie, 2015). While in our study proteins assigned to *Saccharomycetales* fell in numbers with time, a concomitant increase was seen in those from the *Sordariomycetes*, notably *Neurospora crassa,* capable of converting cellulose and hemicellulose to ethanol (Deshpande et al., 1986; Dogaris et al., 2013). As *Saccharomyces* cannot hydrolyse hemi(cellulose) or ferment pentose sugars, they likely thrived while hexose sugars were readily available. Once these were consumed, *Sordariomycetes* abundance increased reflecting their ability to breakdown hemicellulose and ferment pentose sugars. This is particularly relevant considering the high hemicellulose content of the food waste (32%; (Nzeteu et al., 2018)). In addition to *Neurospora crassa*, numerous filamentous *Ascomycota* are known for their prolific cellulase and hemicellulase activity, which make them commonly used in industry (Shah et al., 2017). Such fungal species, including *Trichoderma reesei*, were detected in our protein datasets, as were a number of bacterial species capable of fermenting complex organic matter, including *Clostridium phytofermentans* (Petit et al., 2015) and *C. cellulolyticum* (Desvaux, 2005). *Enterobacteriales* and *Saccharomycetales* were also involved in hydrolysis, with *Enterobacter* previously reported as highly efficient hydrolysers of food waste at low pH (Yan et al., 2014). As *Enterobacteriales* can rapidly respond and survive in acidic pH (Bearson et al., 1997), they might have had a competitive advantage in our acidic reactors. It is worth noting that no cellulase or hemicellulase enzymes were detected. These are typically secreted extracellularly, due to the large nature of their substrates (Collins et al., 2005). As the method employed herein involved recovering microbial cells via differential centrifugation the majority of extracellular proteins would have been lost. Such a method was necessary, however, due to the high protein content from the food waste, several orders of magnitude higher than that of microbial origin, thereby significantly hindering microbial protein identification (Zubarev, 2013).

Acidogenic proteins could mostly be attributed to *Lactobacillales*, which was in agreement with the significant lactic acid production seen on days 1 to 7 of the trial (Fig. 5). This mirrors nucleic acid derived data which identified *Lactobacillus* as amongst the most influential genera (Table S3). *Bacillales* and *Clostridiales* also contributed to acidogenesis. While a few proteins associated specifically with butyrate production were detected, none were detected for caproate production, which mostly occurred after day 7 (Fig. 2). However, *Clostridium* species typically associated with the production of these longer chain fatty acids were identified, including *Clostridium kluyveri* (butyrate, caproate) and *Eubacterium rectale* (butyrate), perhaps involved in butryrate and caproate production seen on days 8 and 9 of the trial (after metaproteomic sampling). A low number of acetogenic proteins were detected and these were mostly assigned to *Ascomycota*, highlighting the role of fungi to food waste AD. Finally, it is worth noting the importance of LAB in our systems, together with the presence of *Saccharomycetales* as synergy between these microbial groups have been previously documented, particularly at low pH (Liu and Tsao, 2009; Lim et al., 2015).

5. Conclusions

We aim to investigate the microbiome underpinning VFA recovery from food waste AD. Butyrate was the most abundant VFA produced in our systems, where low pH prevailed likely resulting from high organic loading rates. Low pH was found to shape microbiome composition with regards to methanogens inhibition, and likely influenced bacterial and fungal dynamics. Food waste hydrolysis was driven by *Ascomycota* and *Proteobacteria*, while VFA accumulation implicated diverse bacterial and fungal species and *Clostridium* likely converted lactate to butyrate. In light of these results, we recommend that fungal bioaugmentation strategy could be explored to further optimise the process.

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Data availability

16SrRNA sequence data were deposited on NCBI's Sequence Read Archive under the accession number PRJNA692345. Metaproteomic

data can be accessed at https://doi.org/10.17630/e2603185-dc54-4034-9c73-bb20749caf60. An interactive view of the chord plot from Fig. 4 is available here: https://cethorn.shinyapps.io/nzeteuetalchordomics/.

CRediT authorship contribution statement

FA and VOF designed the research. CN ran the bioreactors and carried out the corresponding process analyses, as well as the biomolecule co-extraction. AJ prepared all the protein samples for downstreams analyses. SS performed the MS analysis. CN, AJ, CT, KMD and FA analysed and interpreted the data. FA, CN, AJ and CT wrote the paper with input from KMD and VO.

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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