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RESEARCH

Identification of protein-degraders in an anaerobic digester by protein stable isotope probing and metagenomics

Zhe Deng[1] · Jan Struckmann Poulsen[2] · Jeppe Lund Nielsen[2] · David G. Weissbrodt[3,4] · Henri Spanjers[1] · Jules B. van Lier[1]

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Abstract

Presence of carbohydrates hampers protein degradation in anaerobic digesters. To understand this phenomenon, we used proteogenomics to identify the active protein-degraders in the presence of low and high carbohydrates concentrations. Active metabolic pathways of the identified protein-degraders were investigated using proteomics with ^{13}C-protein substrates (protein stable isotope probing). Results showed that 1) *Acinetobacter* was the active protein-degraders under both protein-fed and protein-glucose mixture-fed conditions, 2) the relative abundance of *Acinetobacter* was not affected by the presence of carbohydrates, 3) the incorporation of the ^{13}C-labelled protein substrate was predominantly observed in outer membrane-bound proteins and porin proteins, which are associated with proteinases or the transportation of amino acids across the cell wall. The *Acinetobacter* metabolic model and the incubation conditions suggested that glucose and proteins were degraded through anaerobic respiration. The negative impact of carbohydrates on protein biodegradation was attributed to *Acinetobacter*'s preference for carbohydrates. This work highlights that efficient degradation of protein and carbohydrate mixtures in anaerobic digesters requires a staged or time-phased approach and enrichment of active protein-degraders, offering a new direction for process optimization in anaerobic digestion systems.

Key points

- *Acinetobacter identified for the first time as main anaerobic protein-degrader*
- *Metabolic model revealed protein degradation via anaerobic respiration*
- *Metabolic pathway analysis indicated SO_4^{2-} or Fe^{3+} as terminal electron acceptors*

Keywords Anaerobic digestion · Anaerobic respiration · Protein-degraders · Protein stable isotope probing (protein-SIP) · Metagenomics

Introduction

Microorganisms play a central role in the biological treatment of waste and wastewater. A better understanding of the underlying microbial ecology provides important insights into phenomena observed in the degradation processes (Cerruti et al. 2021). Anaerobic digestion (AD) is a consolidated technology, but still many fundamental questions remain to be addressed. One specific challenge relates to why the presence of carbohydrates negatively affects the anaerobic protein degradation.

Anaerobic protein degradation generally involves three steps, hydrolysis of proteins to amino acids, acidification or deamination of amino acids to ammonium (NH_4^+) and volatile fatty acids (VFA), and finally methanogenesis of VFAs (McInerney 1988). In batch AD processes, proteins were

✉ Zhe Deng
z.deng-2@tudelft.nl

1 Department of Water Management, Delft University of Technology, Stevinweg 1, 2628 CN Delft, Netherlands

2 Section for Biotechnology, Department of Chemistry and Bioscience, Aalborg University, Fredrik Bajers Vej 7H, 9220 Aalborg, Denmark

3 Department of Biotechnology, Delft University of Technology, Van Der Maasweg 9, 2629 HZ Delft, Netherlands

4 Department of Biotechnology and Food Science, Norwegian University of Science and Technology, Sem Sælands Vei 8, 7034 Trondheim, Norway

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observed to be degraded after carbohydrates are depleted, and higher availability of carbohydrates (higher C/N) led to lower protein hydrolysis and amino acids degradation (Wang et al. 2014; Yang et al. 2015; Yu and Fang 2001). Similarly, in continuous-flow AD processes, the presence of carbohydrates retards the anaerobic degradation of proteins as observed in mixed cultures acclimated to proteins and exposed to carbohydrates (Breure et al. 1986). In our previous work, Deng et al. (2022), observed a decrease of 75% in protease activity and of 90% in deamination activity, when lactose was added to the substrate of a bioreactor after 84 days of feeding with only casein. Concomitantly, a community shift was observed, the casein-fed community structure shifted to resemble that of the lactose-fed community. However, 50% of all the identified dominating genera before and after the shift has been described with metabolic traits to degrade carbohydrates and proteins (or amino acids) in the MiDAS (Microbial Database for Activated Sludge) field guide (Dueholm et al. 2021). It was proposed that the competition for substrate utilization by active species, rather than the competition between microbial populations, impairs the anaerobic degradation of proteins. Nonetheless, the identity of the active protein-degraders in a mixed culture from a digester ecosystem remains unsolved. Moreover, the metabolic activity of these protein-degraders, and their impairment by the presence of carbohydrates, are yet to be unveiled.

To address these gaps, identifying the active protein-degraders and understanding their metabolic responses to the presence and absence of carbohydrates can be addressed through methods involving stable isotope probing (SIP) such as DNA-SIP or RNA-SIP, lipid-SIP (Webster et al. 2006), and protein-SIP (de Jonge et al. 2022). SIP methods are based on the labelling of various macromolecules of the metabolically active species through assimilation of typically ^{13}C- or ^{15}N-labelled substrates. The nucleic acids, lipids, or proteins of these active species, subsequently, can be used to identify the organisms actively incorporating these molecules. DNA-SIP and RNA-SIP can provide phylogenetic identities of the active assimilating microorganisms. However, it is challenged by 1) the low mass fraction of DNA (~ 1%) and RNA (~ 5–15%) in cells compared to other cellular constituents such as proteins (~ 30–60%), and 2) the fractionation of partially labelled nucleic acids (Jehmlich et al. 2008). Lipid-SIP can identify the microbial populations of interest (*e.g.*, methane-oxidizing), but only provides a low taxonomic resolution (Jehmlich et al. 2008). For the above reasons, in our present work, we focused on the use of protein-SIP to identify the active microbiome. In fact, the combination of protein-SIP with genome-centric metagenomics is shown to be a powerful approach to link phylogenetic and functional information of active microorganisms (Mosbæk et al. 2016). Since enzymes catalyse biochemical reactions, the identification of actively labelled proteins can provide important information of metabolic processes within the active microorganisms (Jehmlich et al. 2008).

Here, we combined protein-SIP with metagenomics to identify the active protein-degraders in a mixed culture of an anaerobic sequencing batch reactor (AnSBR) treating slaughterhouse wastewater. We compared the identified protein degraders under experimental conditions with low and high carbohydrate concentrations. Finally, the metabolic pathways of the active protein-degraders were investigated. Our work highlights that efficient degradation of protein and carbohydrate mixtures in anaerobic digesters requires a staged or time-phased approach and enrichment of active protein-degraders, offering a new direction for process optimization in anaerobic digestion systems.

Materials and methods

Inoculum and substrates

The inoculum was taken from an AnSBR fed with real slaughterhouse wastewater. The AnSBR setup included a 10-L buffer tank, a 30-L reactor, and a 12-L settling tank. Four cycles were conducted daily, each lasting 6 h. The organic loading rate varied from 2.0 to 6.2 g COD L^{-1} d^{-1}, with a hydraulic retention time ranging from 2.9 to 1.5 days and a solids retention time between 55 and 115 days. Further operational details of the AnSBR can be found in Deng et al. (2024). The inoculum was composed of total suspended solids (TSS) at 13.7 g·L^{-1}, volatile suspended solids (VSS) at 12.4 g·L^{-1}, and ammonium nitrogen (NH_4^+-N) at 0.1 g·L^{-1}. The pH of the inoculum is 7.0 ± 0.1. On average, 52.5 ± 1.0 mL of inoculum was added to each incubation bottle, resulting in an initial biomass concentration of about 2.0 g VSS·L^{-1}. The VSS and NH_4^+-N concentration of the biomass in the incubation bottles are listed in Table 1.

Incubations were performed using a medium rich in proteins, either Unlabelled High Performance OD2 Media solution for *E. coli* (111001402, Silantes, Germany) or labelled ^{13}C High Performance OD2 Media solution for *E. coli* (110201402, Silantes, Germany), as well as glucose (G8270, Merk, the Netherlands) for protein-glucose mixtures. Both OD2 Media solutions were based on a bacterial hydrolysate of *Cupriavidus necator*. More than 98% of the carbon in the ^{13}C High Performance OD2 Media solution for *E. coli* was ^{13}C-labelled. The details of chemical compositions can be found in the supplementary material Table S1. The composition and characteristics of the three substrate media, unlabelled protein, ^{13}C-protein, and mixture of ^{13}C-protein and glucose, are listed in Table 1. These substrate media were used in the UP (unlabelled protein), LP (labelled protein), and LPG (labelled protein and glucose) incubations,

Table 1 Composition and characteristics of the three types of substrate media and characteristics of inoculum used in the UP (unlabelled protein), LP (labelled protein), and LPG (labelled protein and glucose) incubations in this study

Incubations	Substrate Characteristics					Inoculum Characteristics	
	Substrate medium composition	Added in 1 L demineralised water	Initial concentration in the incubation bottles			VSS	NH_4^+-N
			COD	TN	NH_4^+-N		
		(mL)	($mg \cdot L^{-1}$)	($mg \cdot L^{-1}$)	($mg \cdot L^{-1}$)	($g \cdot L^{-1}$)	($mg \cdot L^{-1}$)
UP	unlabelled proteins	[a]120.0 ± 0.0	717 ± 0.1	72 ± 0.0	13 ± 0.0	2.2 ± 0.07	18 ± 0.5
LP	^{13}C-proteins	[b]190.0 ± 0.0	717 ± 0.0	72 ± 0.0	9 ± 0.0	2.1 ± 0.0	17 ± 0.0
LPG	^{13}C-proteins and glucose	[b,c]190.0 ± 0.1	1334 ± 0.0	133 ± 0.1	75 ± 0.0	2.1 ± 0.0	17 ± 0.0

[a] Unlabelled High Performance OD2 Media solution; [b] ^{13}C High Performance OD2 Media solution; [c] 280 $mg \cdot L^{-1}$ NH_4Cl was added to adjust the COD/N (chemical oxygen demand/nitrogen) ratio to 10. 250 mg $NaHCO_3$ was added into UP and LP substrate media, 700 mg was added into the LPG medium. TN: total nitrogen; VSS: volatile suspended solids

respectively. The content of glucose was less than 30 mg in 1000 mL substrate media. Before starting the incubation, the pH of the substrate media was adjusted to 6.0 ± 0.1 by adding drops of 0.1 $mol \cdot L^{-1}$ NaOH or HCl, and buffered by adding $NaHCO_3$ (144–55 - 8, Merk, the Netherlands) and flushing N_2/CO_2 gas (N_2:CO_2 = 70%:30%) into the liquid for 2 min.

Anaerobic incubation

The incubation was carried out in triplicates using 500 mL glass bottles (Z674400, Duran®, the Netherlands) with a working volume of 300 mL and a headspace of 200 mL. Inoculum was first added into the bottles, followed by the substrate medium, and then the bottles were closed with a lid GL 45 with twin connectors (01–0206 - 02, BPC Instruments AB, Sweden). A tube (6.4/3.2 mm, Tygon® Tubing, BPC Instruments AB, Sweden) connected to the inlet was submerged in the liquid and used for sampling of the liquid phase; a tube connecting to the outlet was used for gas collection. A photo of the setup and a schematic diagram of the incubation bottle are given in the supplementary material Fig. S1. Before incubation, N_2/CO_2 gas was used to flush the bottles to establish anaerobic conditions through the tube connecting to the inlet. The bottles were then incubated over 96 h in a water bath at 25 °C and continuously stirred at 100 rpm. The time point 0 h was set when the temperature in the reactor reached 25 °C, which was 30 min after the incubation bottles were placed into the water bath.

Mixed liquor samples of 2.5 mL were collected from the incubation bottles with a syringe (307,731, BD, USA) at 0, 1, 2, 4, 6, 9, 24, 30, 48, 72, and 96 h, to analyse the pH, total nitrogen (TN), NH_4^+-N, VFAs, and the total (TCOD) and soluble (SCOD) concentrations of chemical oxygen demand (COD). The pH was measured with a pH sensor Multi 9420, WTW inoLab®, Germany). Mixed liquor samples (1 mL and 0.5 mL) were stored at − 20 °C pending proteins and DNA extraction, respectively. An additional 1 mL of each mixed liquor sample was stored at 4 °C and analysed for the aforementioned chemical constituents on the same day as when the samples were collected. The sample conditioning and preparation of the analyses for chemical analyses are described hereafter.

Basic performance parameters

The TCOD of the samples was measured with Hach kits (LCK214 and LCK514, Merck, Germany) without any pre-treatment. For the measurement of TN, NH_4^+-N and SCOD with Hach kits (LCK338, LCK303, and LCK214, Merck, Germany), the samples were first centrifuged at 13,000 × g for 5 min, and then filtered through a 0.45-µm polypropylene membrane filter (WHA68782504, Whatman®, UK). The same pre-treatment steps were also done before measuring VFAs. The composition of VFAs, including C2 (acetic acid), C3 (propionic acid), C4 (butyric acid), iC4 (iso-butyric acid), C5 (valeric acid), iC5 (iso-valeric acid) and iC6 (iso-caproic acid), was analysed using a gas chromatograph (7820 A, Agilent Technologies, the Netherlands) equipped with a flame ionization detector using a CP 7614 column (WCOT fused Silica 25 m × 0.55 mm, CP-wax 58 FFAP capillary, Agilent Technologies, the Netherlands). The temperature of the injector was 250 °C. Dinitrogen carrier gas was supplied at 28.5 $mL \cdot min^{-1}$ with a split ratio of 10. The GC temperature gradient was started at 100 °C held for 2 min and increased to 140 °C at 7 $°C \cdot min^{-1}$ and held for 6 min at this temperature.

V3-V4 16S rRNA gene amplicon sequencing

The 0.5 mL mixed liquor samples taken at the beginning (0 h) and end (96 h) of the experiment were used for amplicon sequencing to characterise their bacterial community compositions. DNA was extracted with FastDNA™ Spin Kits for Soil (MP BIOMEDICALS, USA). The concentrations of the DNA extracts were measured using a Qubit 3 Fluorometer (Thermo Fisher Scientific, USA). The DNA extracts with a concentration higher than 20 ng·μL^{-1} were then sent for V3-V4 16S rRNA gene amplicon sequencing on an Illumina NovaSeq 6000 platform (Novogene, UK). The amplicon libraries were generated using the forward and reverse primers 341 F [(5′–3′) CCTAYGGGRBGCASCAG] and 806R [(5′–3′) GGACTACNNGGGTATCTAAT] for bacteria/archaea in the V3–V4 hypervariable regions of the 16S rRNA gene (Novogene, UK). Paired-end reads (2 × 250 bp) were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. Paired-end reads were merged using FLASH (V1.2.7) (Magoč and Salzberg 2011), and the splicing sequences were filtered using QIIME (V1.7.0) to obtain high-quality clean tags (Caporaso et al. 2010). The effective tags were obtained by using the UCHIME algorithm to detect and remove chimeric sequences (Edgar et al. 2011). The sequences of the effective tags were analysed with Uparse (V7.0.1001) (Edgar 2013). Sequences with ≥ 97% similarity were assigned to the same Operational Taxonomic Unit (OTU) and screened for further annotation by the Mothur software against the SSUrRNA database of the SILVA Database (Wang et al. 2007). Phylogenetic relationship of all OTUs were obtained using MUSCLE (Version 3.8.31) (Edgar 2004). OTUs abundance information were normalised using a standard of sequence number corresponding to the sample with the least sequences. The amplicon sequencing results were visualised using the ampvis2 (Andersen et al. 2018) and ggplot2 (Wickham et al. 2016) packages in the R software (V 2.15.3). The sequences were deposited at DDBJ/EMBL/GenBank under the accession numbers OP474180-OP476210.

Metagenomics and bioinformatics

Three metagenomes were prepared from the inoculum before incubation, as references for the mapping of the metaproteomes. DNA was extracted using the FastDNA™ Spin Kit for Soil (MP Biomedicals, USA), following the manufacturer's recommendations. The DNA extracts were commercially sequenced by Novogene (UK) on an Illumina NovaSeq 6000 platform. Pair-end reads with a length of 250 bp were generated, the sequencing depth was at least 40 million read pairs per sample. The pre-processing, trimming, filtering, and assembling of the metagenome was conducted as previously described (Poulsen et al. 2022). Briefly, the raw reads were pre-processed by PhiX sequence control, trimmed for adaptors, and quality filtered for a minimum Phred score of 20, which corresponds to a 99% base call accuracy, ensuring a comprehensive dataset while maintain reliable sequence accuracy. The trimmed reads were assembled applying k-mers of 21, 33, 55, and 77, and contigs shorter than 1000 bp were removed. The mmgenome2 package (https://kasperskytte.github.io/mmgenome2/) was used in R to generate a prediction of open reading frames in the metagenome, identify the essential genes, taxonomically classify the contigs, and locate the labelled bins and the known protein-degraders, resulting in the obtaining of their metagenome-assembled genomes (MAGs). The three obtained MAGs were deposited in DDBJ/EMBL/GenBank under the accession number SRR30024180-SRR30024182. Known protein-degraders within bacteria and archaea containing protease coding genes were identified according to the UniprotKB database (Zaru et al. 2020) and literature.

Protein extraction and protein-SIP analysis

Proteins were extracted using phenol liquid–liquid extraction as previously described (Poulsen et al. 2022), followed by in-gel digestion (Mosbæk et al. 2016). The desalting and analysis of tryptic peptides by automated liquid chromatograph-electrospray ionisation tandem mass spectrometry (LC–ESI–MS/MS) were performed as previously described (Poulsen et al. 2021). The MetaProSIP tool (Sachsenberg et al. 2015) was used to analyse the proteomic data in an OpenMS pipeline, and the search database metaproteome was generated from the metagenome and annotated using Prokka (v.1.14), as previously described (Poulsen et al. 2022).

Metabolic pathway analysis of active degraders of proteins

The metabolic pathway of the identified active protein degraders was analysed from its MAG. A narrative was built in KBase to this end (Arkin et al. 2018). The DNA contigs related to the active protein degraders were extracted from one of the three MAGs. The extracted active protein degraders contigs were then binned to produce a single-specie MAG, using the algorithm "Extract Bins as Assemblies from BinnedContigs" (v1.1.1). The MAG was checked by using "Assess Genome Quality with CheckM" (v1.0.18), and then annotated using "Annotate Microbial Assembly with RASTtk" (v1.073). The MAG was compared with 30 complete reference genomes (NCBI database) using "Insert Set of Genomes into SpeciesTree" (v2.2.0). The function profile of MAG was further visualised using "View Function Profile for Genomes" (v1.4.0). The metabolic pathway was analysed by building a metabolic model using "Gapfill Metabolic Model".

Results

Comparison of the protein degradation performance and the microbial community compositions in the UP, LP and LPG incubations

Protein degradation in the presence of low and high concentrations of carbohydrates

The evolutions of the concentrations of total (TCOD) and soluble (SCOD) COD-equivalents normalised by their initial concentrations, of the concentrations of the ammonium (NH_4^+-N) produced as percentage of the initial total nitrogen (TN) (*i.e.*, NH_4^+/TN ratio), and of the pH-value during the anaerobic batch incubations performed with unlabelled proteins (UP), labelled proteins (LP) and a mixture of labelled proteins and glucose (LPG) are presented in Fig. 1.

On average, the normalised TCOD in the three incubations with UP, LP, and LPG was removed by a percentage of 20% ± 7% (Fig. 1a). The normalised SCOD decreased by 70% ± 12% (Fig. 1b). In concentration terms, the decrease in TCOD (1100 ± 330 mg·L^{-1}) was similar to that of SCOD (1060 ± 75 mg·L^{-1}) in LPG. In UP and LP, the decrease in SCOD (460 ± 60 mg·L^{-1} and 530 ± 50 mg·L^{-1}, respectively) was about 50% of the decrease in TCOD (890 ± 150 mg·L^{-1} and 1030 ± 220 mg·L^{-1}, respectively). The smaller decrease in SCOD is likely attributed to reduced measurement accuracy at low SCOD concentrations, because the NH_4^+-N/TN ratio in the UP and LP incubations increased to as high as 90 ± 6% after 96 h (Fig. 1c). This suggests that the proteins supplied in the UP and LP media were efficiently converted and deaminated. Whereas with LPG, the NH_4^+-N/TN reached only a partial conversion ratio of 75 ± 1% after 96 h (Fig. 1c). Assuming a COD consumption of 5% for bacterial growth under anaerobic conditions (van Lier et al. 2020) and a COD:N molar ratio of 5 needed to form bacterial biomass (Münch and Pollard 1997), a mass of 3 mg of nitrogen was theoretically assimilated by microorganisms in the LPG incubation. Based on this assumption, there was still a gap of 9 mg N between the amount of produced NH_4^+-N and the added TN in the LPG. This gap indicated that the added protein was not fully converted in the presence of high glucose concentration. The pH was maintained between 6.0 and 6.5 during the incubation experiment (Fig. 1d). Excluding other factors, the expected pH increase caused by the production of NH_4^+ shall be approximately 2.4 based on

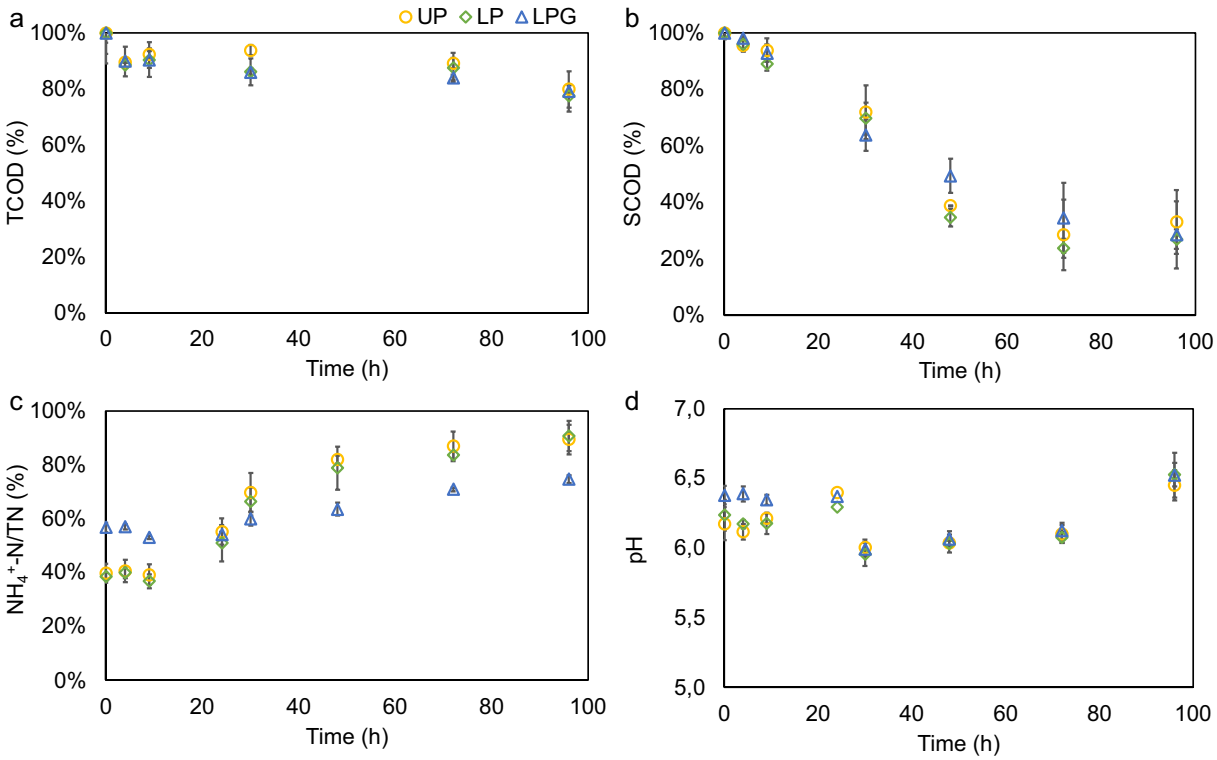


Fig. 1 Substrate concentrations and pH during the 96-h incubation period. **a**) Total COD (% of initial TCOD concentration); **b**) Soluble COD (% of the initial SCOD concentration); **c**) Conversion ratio of ammonium nitrogen from total nitrogen (NH_4^+-N/TN) indicating the NH_4^+-N production as % of the added TN; **d**) pH evolutions. All data points are presented as averages and standard deviations from triplicates. Legends of incubations: yellow circle – unlabelled proteins (UP), green diamond – ^{13}C-labelled proteins (LP), blue triangle – mixture of ^{13}C-labelled proteins and glucose (LPG)

a Phylum

	0h			96h		
	LP	LPG	UP	LP	LPG	UP
Proteobacteria	28.5	26.7	28.6	26.4	27.1	29.3
Cloacimonetes	21.3	20.1	19	20	17.7	16.1
Firmicutes	11.7	11.6	11	13.3	15.5	15.4
Bacteroidetes	13.1	12.9	13.2	11.5	12.1	11.8
Chloroflexi	8.9	10.4	10.8	10.3	10.7	10.8
Euryarchaeota	8.4	10.7	9.8	9.3	9.5	8.9
Thermotogae	1.3	1.5	1.4	2.3	1.5	1.5
Spirochaetes	1.4	1.3	1.5	1	1	1.1
Campylobacterota	1.5	1.1	0.7	2	1.2	0.8
Synergistetes	0.7	0.7	0.7	0.9	0.9	0.9
Verrucomicrobia	0.6	0.5	0.6	0.5	0.5	0.6
Atribacteria	0.3	0.3	0.3	0.3	0.3	0.3
Kiritimatiellaeota	0.3	0.2	0.2	0.2	0.2	0.2
Planctomycetes	0.2	0.2	0.2	0.2	0.1	0.2
Calditrichaeota	0.2	0.2	0.2	0.2	0.2	0.2

b Genus

	0h			96h		
	LP	LPG	UP	LP	LPG	UP
Candidatus_Cloacimonas	20.1	19	18	18.9	16.8	15.2
unidentified_Anaerolineaceae	6.5	7.7	8	7.7	8.1	8.1
Acinetobacter	5.9	4.8	5.5	6.4	5.9	7.2
Smithella	5.3	5.4	5.3	4.8	4.8	4.7
Methanosaeta	4.4	5.8	5.2	4.6	5.1	4.6
Methanobacterium	3.3	4	3.7	3.8	3.6	3.5
unidentified_Clostridiales	2.9	2.8	2.8	3.6	4.7	4.8
o__Bacteroidales_OTU_6	2.5	2.4	2.4	2.3	2.5	2.4
o__Bacteroidales_OTU_7	2.3	2.3	2.4	1.9	2	1.9
Syntrophorhabdus	1.8	1.8	1.9	1.7	1.6	1.8
Mesotoga	1.3	1.5	1.4	2.3	1.5	1.5
f__Syntrophaceae_OTU_16	1.5	1.5	1.5	1.2	1.2	1.3
Denitratisoma	1.5	1.4	1.5	1.2	1.2	1.3
f__Lentimicrobiaceae_OTU_9	1.4	1.4	1.4	1.3	1.3	1.3
f__Prolixibacteraceae_OTU_10	1.4	1.4	1.4	1.2	1.3	1.1

◂**Fig. 2** Evolutions of the bacterial community compositions over the incubation period of 96 h. **a**) top 15 phyla and **b**) top 15 genera in UP, LP, and LPG incubations. V3-V4 amplicon sequencing profiles were analysed at 0 h and 96 h. The relative abundances of operational taxonomic units (OTUs) displayed on the heatmaps are averages calculated on triplicate incubation. The maximum relative standard deviation on the amplicon sequencing measurements was 0.02%

the NH_4^+/NH_3 equilibrium. However, this pH increase is buffered by the production of CO_2 during the degradation of both amino acids and glucose, overall resulting in a negligible pH increase.

Bacterial community composition

The effect of substrate conditions on the bacterial community composition was investigated by V3-V4 16S rRNA gene amplicon sequencing for the three incubations (UP, LP, LPG). In total, 1,271,436 reads were obtained from 18 samples. The minimum count of sequences obtained per sample was 61,187, the maximum count obtained was 77,097, and the median was 72,416. The community profile of the sample taken at 0 h, which was 30 min after the bottles were placed into the water bath, was comparable at both phylum and genus levels in all bottles and did neither vary significantly between the different treatments nor during the incubation periods of 96 h (Fig. 2).

The phyla of *Proteobacteria* (relative abundance of 26–29% of OTUs) and *Cloacimonetes* (16–21%) were predominant in the microbiomes of all incubations, regardless of the substrates supplied (Fig. 2a). Minor Phyla of *Firmicutes*, *Bacteroidetes*, *Chloroflexi, Euryarchaeota*, *Thermotogae*, *Spirochaetes* and *Campylobacteriota* were present at a relative abundance above 1%. The results matched with previous studies: *Chloroflexi*, *Proteobacteria,* and *Bacteroidetes* were reported dominant in a casein-fed reactor (Deng et al. 2022); *Firmicutes* in a reactor fed with bovine serum album (Tang et al. 2005); and *Firmicutes, Proteobacteria,* and *Bacteroidetes* in an anaerobic reactor treating slaughterhouse wastewater (Jabari et al. 2016).

Figure 2b displays the top 15 genera in each incubation, whereas the relative abundance of the top 15 genera in each bottle are shown in supplementary material Fig. S3. *Acinetobacter* increased by 0.5—1.7% in averaged relative abundance, an unidentified *Clostridiales* by 0.7–2.0%, whereas "*Candidatus* Cloacimonas" decreased by 1.2—2.8% over the incubation period of 96 h. Populations of the order *Clostridiales* are reported degraders of amino acids (McInerney 1988), while "*Ca.* Cloacimonas acidaminovorans" is known to degrade amino acids in an anaerobic fed-batch reactor supplied with casein (Kovács et al. 2013). Notably, these averaged variations do not necessarily indicate the change of community structure, but a potential predominancy of active protein-degraders.

Metagenome analysis

The total number of paired-end sequences used to build the metagenome was 172,172,549 bp per sample. When assembled, it resulted in a de novo metagenome assembly of 1,242,870,255 bp divided in 368,064 contigs. Half of the metagenome assembly was covered by contigs larger than or equal to 5,650 bp (N50 value). The metagenome assembly consisted of 82.6% of *Bacteria* and 17.3% of *Archaea,* eukaryotic and viral sequences were removed from the dataset. Among the 36 identified phyla, *Proteobacteria* were the most abundant representatives with 51% of all identified scaffolds. The microbial composition of the metagenome was in accordance with the 16S rRNA gene amplicon sequencing profiles. A comparison of the metagenome profile and amplicon sequencing profile is provided in the supplementary material Fig. S4.

Identification of protein-degraders

Protein-degraders identified in the LP incubation

The protein pools extracted from biomass samples, collected after 0, 6, 24 and 48 h of the LP incubation involving ^{13}C-labelled proteins as substrate, were analysed for protein-SIP by MetaProSIP. The ^{13}C-labelled proteins as substrate were a hydrolysate of *Cupriavidus necator*, containing mainly proteins and amino acids, and less than 30 $mg{\cdot}L^{-1}$ glucose. A detailed composition of the substrate can be found in Table 1 and supplementary materials Table S1.

Table 2 lists the number of identified proteins that incorporated the ^{13}C-label at the different time instants, in the column labelled protein ID. The number of reference proteins (NCBI database) that have an almost identical match (> 99%) to the detected proteins are given in the column Hits, and the functional and phylogenetic annotation of the reference proteins are provided as well. The ^{13}C-labelled substrate was found to be incorporated into biomass proteins after 6 h, and the number of labelled proteins increased over time. After 6 h, three labelled proteins were detected: all were assigned to the phylum *Proteobacteria*, of which two affiliated with the genus *Acinetobacter* and one to *Klebsiella*. After 24 h, ten labelled proteins were detected, of which nine could be assigned to the genus *Acinetobacter*. After 48 h, fifteen labelled proteins were detected; two could not be annotated and all others were assigned to the genus *Acinetobacter*. This highlighted the preponderance of populations of *Acinetobacter* as protein-degraders. Based on literature, the substrate utilization properties of the labelled genera detected in our study are summarised (Table S2). The genus *Acinetobacter* was the most frequently identified. *Acinetobacter* is capable of degrading proteins and sugars under aerobic conditions, and utilising accumulated polyphosphates

to survive under anaerobic conditions (Dueholm et al. 2021; Pulami et al. 2023).

Most of the proteins incorporated the ^{13}C-label were identified as outer membrane proteins or porin proteins; their function has generally been considered for the transportation or diffusion of molecules (Welte et al. 1995). One protein was identified as an elongation factor Ts. It associates with the EF-TuGDP complex and induces the exchange of guanosine diphosphate to guanosine triphosphate (Xin et al. 1995). The time-resolved protein-SIP confirmed the uptake of the ^{13}C-labelled protein substrate in the identified microorganisms.

Protein-degraders identified in the LPG incubation

Table 3 lists the identified proteins having incorporated ^{13}C-label from the proteinaceous substrate in the presence of glucose (LPG). In the LPG incubation, the ^{13}C-labelled substrate was found to be incorporated into microorganisms as early as at time 0 h (*i.e.*, 30 min after the incubation bottles were placed into the 25 °C water bath). Three labelled proteins were identified at 0 h and assigned to the genus *Acinetobacter*. Compared to the LP incubation, the incorporation of ^{13}C-labelled substrate into microorganisms occurred earlier in the LPG incubation. According to the kinetic study of Deng et al. (2023), the presence of glucose increased the protein hydrolysis rate by 1.6—1.7 times. Glucose might provide energy for the facultative protein degraders to produce protease, and lead to a faster incorporation of ^{13}C in the LPG incubation.

After 6 h, only one protein was labelled; its sequence could not be annotated (hypothetical protein). The number of labelled proteins peaked after 24 h, with seven proteins having incorporated ^{13}C. Most of the labelled proteins were assigned to the proteobacterial genera *Acinetobacter*, *Escherichia*, *Pluralibacter* or *Lysobacter*. The genus level annotation of some labelled proteins was difficult due to the close genetic relationships among genera *Escherichia*, *Salmonella* and *Shigella* (Silverman et al., 2006). One protein was assigned to the phylum of *Firmicutes*, further related to either the genus *Christensenella* or *Clostridium*. *Clostridia* are known to degrade amino acids and have been reported to dominate the microbiome of an anaerobic reactor fed with bovine serum albumin (Tang et al. 2005). After 48 h, only two labelled proteins were detected and were assigned to the proteobacterial genera *Escherichia* or *Lysobacter* and *Xanthomonas*, respectively. Similar to the LP incubation, most of the labelled proteins in the LPG incubation were identified as outer membrane proteins or porin proteins. In addition, *Acinetobacter* and *Escherichia* were

Table 2 Identified proteins incorporated the ^{13}C-label supplied by the proteinaceous substrate in the LP incubation

Sample Time	Labelled protein ID	Hits	Protein	Phylum	Genus
6 h	**1**	**1**	Elongation factor Ts	*Proteobacteria*	*Acinetobacter*
	2	**1**	outer membrane protein	*Proteobacteria*	*Acinetobacter*
		1	outer membrane protein Omp38		
		7	OmpA family protein		
	3	**2**	OmpK36	*Proteobacteria*	*Klebsiella*
24 h	**1**	**1**	hypothetical protein	*Proteobacteria*	*Acinetobacter*
		1	choice-of-anchor F family protein		
	2	**1**	fimbrial protein	*Proteobacteria*	*Klebsiella*
	3	**1**	hypothetical protein	*Proteobacteria*	
	4	**1**	Elongation factor Ts	*Proteobacteria*	*Acinetobacter*
	5	**1**	outer membrane protein HMP	*Proteobacteria*	*Acinetobacter*
		1	hypothetical protein		
		1	outer membrane protein Omp38		
		11	OmpA family protein		
	6	**1**	outer membrane protein	*Proteobacteria*	*Acinetobacter*
		1	hypothetical protein		
		1	outer membrane protein Omp38		
		7	OmpA family protein		
	7	**1**	hypothetical protein	*Proteobacteria*	*Acinetobacter*
		1	outer membrane protein Omp38		
		5	OmpA family protein		
	8	**1**	OmpA family protein	*Proteobacteria*	*Acinetobacter*
	9	**1**	OmpA family protein	*Proteobacteria*	*Acinetobacter*
	10	**1**	hypothetical protein	*Proteobacteria*	*Acinetobacter*

Table 2 (continued)

Sample Time	Labelled protein ID	Hits	Protein	Phylum	Genus
48 h	**1**	**1**	outer membrane protein A	*Proteobacteria*	*Salmonella*
		1	outer membrane protein A		*Shigella*
		2	outer membrane protein A		*Escherichia*
		1	OmpA/MotB domain protein		*Pluralibacter*
		1	porin OmpA		*Mangrovibacter*
		1	porin OmpA	*Firmicutes*	*Staphylococcus*
		2	porin OmpA	*Proteobacteria*	*Escherichia*
		1	porin OmpA		*Klebsiella*
		1	porin OmpA		*Enterobacter*
	2	**1**	hypothetical protein	*Proteobacteria*	*Syntrophus*
	3	**1**	hypothetical protein	*Proteobacteria*	*Acinetobacter*
		1	choice-of-anchor F family protein		
	4	**1**	fimbrial protein	*Proteobacteria*	*Klebsiella*
	5	**1**	hypothetical protein		
	6	**1**	OmpA family protein	*Proteobacteria*	*Acinetobacter*
	7	**1**	outer membrane protein HMP		*Acinetobacter*
		1	hypothetical protein		
		1	outer membrane protein Omp38		
		11	OmpA family protein		
	8	**1**	outer membrane protein	*Proteobacteria*	*Acinetobacter*
		1	hypothetical protein		
		1	outer membrane protein Omp38		
		7	OmpA family protein		
	9	**1**	hypothetical protein	*Proteobacteria*	*Acinetobacter*
	10	**1**	hypothetical protein	*Proteobacteria*	*Acinetobacter*
		1	choice-of-anchor F family protein		
	11	**1**	hypothetical protein	*Proteobacteria*	*Acinetobacter*
		1	outer membrane protein Omp38		
		5	OmpA family protein		
	12	**1**	OmpA family protein	*Proteobacteria*	*Acinetobacter*
	13	**1**	OmpA family protein	*Proteobacteria*	*Acinetobacter*
	14	**1**	hypothetical protein	*Proteobacteria*	*Acinetobacter*
	15	**1**	hypothetical protein	*Proteobacteria*	*Pulveribacter*
		1	hypothetical protein		*Polaromonas*

Samples were measured for protein-SIP after 0, 6, 24 and 48 h. The number of reference proteins with over 99% matching identity (Hits) are given, together with their functional and phylogenetic annotations (at phylum and genus level). Proteins that incorporated ^{13}C-label was not detected at 0 h

the most frequently identified genera that metabolised the ^{13}C-labelled protein substrate (Table S3). Both populations are capable of degrading proteins and sugars (Dueholm et al. 2021). Additionally, although genera *Escherichia and Klebsiella* were frequently identified in both LP and LPG incubations, they were not detected in the amplicon sequencing data. These genera are known to be present in slaughterhouse wastewaters (Savin et al. 2021, 2022). The taxonomic

Table 3 Identified proteins incorporated the ^{13}C-label supplied by the proteinaceous substrate in the LPG incubation

Sample Time	Labelled protein ID	Hits	Protein	Phylum	Genus
0 h	**1**	**1**	outer membrane protein	*Proteobacteria*	*Acinetobacter*
		2	Outer membrane protein Omp38		
		7	OmpA family protein		
	2	**2**	Outer membrane protein Omp38	*Proteobacteria*	*Acinetobacter*
		5	OmpA family protein		
	3	**2**	Outer membrane protein Omp38	*Proteobacteria*	*Acinetobacter*
		10	OmpA family protein		
6 h	**1**	**1**	hypothetical protein		
24 h	**1**	**1**	outer membrane protein II	*Proteobacteria*	*Escherichia*
		1	outer membrane protein A precursor		
		1	outer membrane protein A		
		1	Unclassified		*Lysobacter*
		2	porin OmpA		*Escherichia*
	2	**1**	outer membrane protein II	*Proteobacteria*	*Escherichia*
		1	outer membrane protein A precursor		
		1	outer membrane protein A		
		2	OmpA/MotB domain protein		*Pluralibacter*
		1	porin OmpA		*Escherichia*
	3	**1**	Unclassified	*Firmicutes*	*Christensenella*
		1	Unclassified		*Clostridium*
	4	**1**	outer membrane protein II	*Proteobacteria*	*Escherichia*
		1	outer membrane protein A		
		2	porin OmpA		
	5	**1**	porin OmpA	*Proteobacteria*	*Escherichia*
		1	Unclassified		*Acinetobacter*
		1	outer membrane protein Omp38		
		6	OmpA family protein		
	6	**1**	outer membrane protein II	*Proteobacteria*	*Escherichia*
		2	outer membrane protein A		
		2	porin OmpA		
		1	Unclassified		*Lysobacter*
24 h	**7**	**1**	outer membrane protein II	*Proteobacteria*	*Escherichia*
		1	outer membrane protein II		*Shimwellia*
		1	outer membrane protein II		*Citrobacter*
		1	outer membrane protein A		*Salmonella*
		1	outer membrane protein A		*Shigella*
		2	outer membrane protein A		*Escherichia*
		1	outer membrane protein A		*Serratia*
		1	membrane protein		*Serratia*
		1	porin OmpA		*Escherichia*
		1	porin OmpA		*Klebsiella*
		1	porin OmpA		*Enterobacter*
48 h	**1**	**1**	outer membrane protein II	*Proteobacteria*	*Escherichia*
		2	outer membrane protein A		
		1	Unclassified		*Lysobacter*
		2	porin OmpA		*Escherichia*
	2	**1**	Unclassified	*Proteobacteria*	*Xanthomonas*

Samples were measured for protein-SIP after 0, 6, 24 and 48 h. The number of reference proteins with over 99% matching identity (Hits) are given, together with their functional and phylogenetic annotations (at phylum and genus level)

affiliation by proteomics still faces limitations due to the lack of a comprehensive database for anaerobic digestion (AD), which may lead to insufficient identification.

Acinetobacter was the main protein-degraders

Figure 3a displays the scaffold coverage and GC-content of the metagenome of the microbial community, overlaid by the protein-SIP data. The eight scaffolds holding the labelled protein detected in the LP incubation are highlighted in yellow, whereas the fourteen scaffolds holding the labelled protein detected in the LPG incubation are highlighted in red. The phyla *Firmicutes* and *Proteobacteria*, to which the scaffolds are affiliated, are coloured. The taxonomical classification of the labelled contigs indicated that *Acinetobacter* was the active protein-degrader in the communities of the incubations conducted in both low and high concentrations of glucose. According to the community profiles, *Acinetobacter* and an unclassified *Clostridiales* were selected over the 96 h of incubation (Fig. 2b), supporting the preponderance of these organisms.

According to the UniProt database and literature, the known protein-degraders that hold a protease were identified from the metagenome of the incubated biomass (Fig. 3b). A detailed list of the genera is given in Table S4 in supplementary information.

Possible metabolic pathway of *Acinetobacter* under anaerobic conditions

To investigate the metabolic pathway coded in the genome of *Acinetobacter* for the biodegradation of proteins, a metabolic model was built using the MAG of *Acinetobacter* that was extracted from the sequenced metagenome. The *Acinetobacter* MAG had a 14.5% completeness and 0.0% contamination. We re-annotated the *Acinetobacter* MAG and compared it with 30 complete reference genomes of *Acinetobacter* species downloaded from NCBI database (supplementary Fig. S5).

As shown in Fig. 4, the valine (Val), leucine (Leu), and isoleucine (Ile) are degraded by *Acinetobacter* through the TCA cycle. The pathway for fermentation of VFAs was not found in the built *Acinetobacter* metabolic model. This may be attributed to the 14.5% completeness of the MAG. However, the VFAs fermentation pathway was neither identified in the metabolic model constructed from the complete genome of the closest species, *Acinetobacter brisouii*. The metabolism of fatty acids leads to the production of acetyl-CoA instead of VFAs, which may explain the low VFA concentration during the incubations (supplementary Fig. S2). The TCA cycle provides energy to organisms through oxidation of acetyl-CoA derived from carbohydrates, proteins, and fatty acids. The TCA cycle is involved through respiration (Thauer 1988).

Discussion

Acinetobacter vs known protein-degraders

Acinetobacter is generally regarded as obligate aerobic, often present in anaerobic–aerobic SBR operated for biological removal of organic matter, phosphorus and nitrogen from wastewater (Shelly et al. 2021). This genus exhibits selective growth when the readily biodegradable substrate was leaking from the anaerobic to the aerobic phase of a granular sludge SBR (Weissbrodt et al. 2012). The species *Acinetobacter baumannii* DT (Oanh et al. 2020) and *Acinetobacter* H12 (Ali et al. 2021) can survive under conditions without air supply.

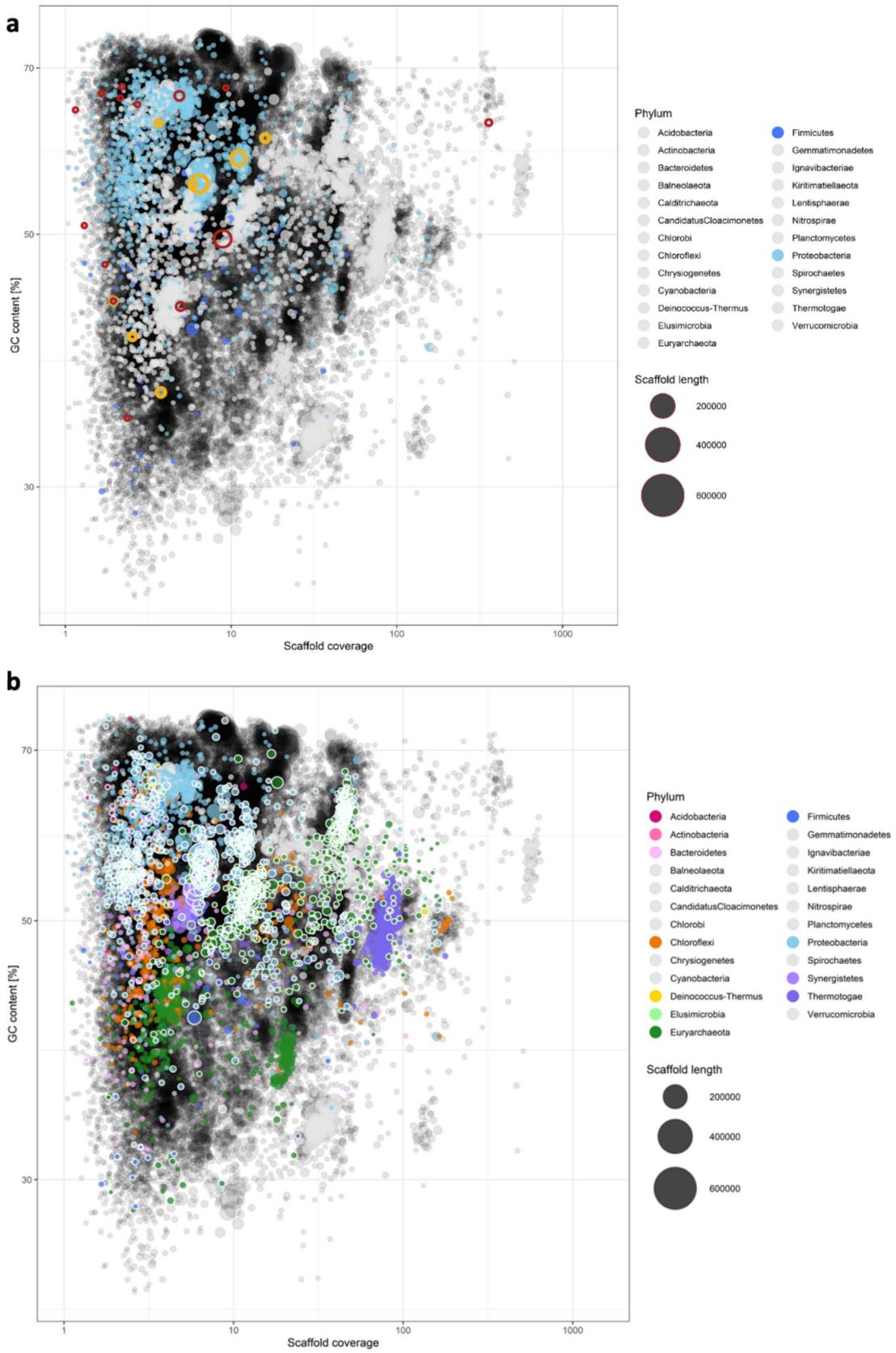
In previous studies, the genera *Clostridium* (McInerney, 1988), *Peptostrptococcus* (Dürre et al., 1983), *Acidaminobacter* (Stams and Hansen, 1984), *Aminomonas* (Baena et al., 1999), *Thermanaerovibrio* (Nanninga et al., 1987), *Sporanaerobacter* (Hernandez-Eugenio et al., 2002), *Sedimentibacter* (Breitenstein et al., 2002), *Aminobacterium* (Baena et al., 2000), *Tissierella*, and *Lutispora* (Chen et al., 2018) have been able to ferment amino acids or proteins. Most of the known anaerobic degraders of amino acids belong to the phylum *Firmicutes* (Fig. 3b). In addition, the genus *Bacteroides* (phylum of *Bacteroidetes*), and the genera *Streptococcus*, *Clostridium*, *Bacillus* and *Staphylococcus* (phylum of *Firmicutes*) are protein-degraders in the human gut (Macfarlane et al. 1986).

Although *Acinetobacter* is one of the genera that harbour proteases (Table S4), it has been seldom reported as the main protein degrader in AD. The identification of *Acinetobacter* as the main protein degrader in our study is therefore interesting and intriguing.

Anaerobic degradation of proteins by *Acinetobacter*

The function of the outer membrane protein and porin membrane can possess a cell envelope-associated protease, which hydrolyse proteinaceous substrates on the surface of the cell and facilitate transport of the formed peptides into the cell (Sadat-Mekmene et al. 2011). The cell envelope-bounding protease activity has been reported in *Acinetobacter calcoaceticus* under aerobic conditions (Fricke et al. 1987); and the growth and respiratory efficiency of *Acinetobacter calcoaceticus* is reported to be unaffected by oxygen concentration in the range of 0.0–0.8 $mg{\cdot}L^{-1}$ (Hardy and Dawes 1985). However, *Acinetobacter* can survive under anaerobic conditions by utilizing the accumulated polyphosphates as a phosphorus reserve, but thus far, no growth of the population was observed (Pulami et al. 2021). The metabolism of protein degradation by *Acinetobacter*, whether via fermentation or anaerobic respiration was still unclear.

Known terminal acceptors involved in anaerobic respiration include organic compounds, *i.e.*, fumarate, dimethyl


a
GC content [%]
70
50
30
1
10
100
1000
Scaffold coverage
Phylum
Acidobacteria
Actinobacteria
Bacteroidetes
Balneolaeota
Calditrichaeota
CandidatusCloacimonetes
Chlorobi
Chloroflexi
Chrysiogenetes
Cyanobacteria
Deinococcus-Thermus
Elusimicrobia
Euryarchaeota
Firmicutes
Gemmatimonadetes
Ignavibacteriae
Kiritimatiellaeota
Lentisphaerae
Nitrospirae
Planctomycetes
Proteobacteria
Spirochaetes
Synergistetes
Thermotogae
Verrucomicrobia
Scaffold length
200000
400000
600000
b
GC content [%]
70
50
30
1
10
100
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Scaffold coverage
Phylum
Acidobacteria
Actinobacteria
Bacteroidetes
Balneolaeota
Calditrichaeota
CandidatusCloacimonetes
Chlorobi
Chloroflexi
Chrysiogenetes
Cyanobacteria
Deinococcus-Thermus
Elusimicrobia
Euryarchaeota
Firmicutes
Gemmatimonadetes
Ignavibacteriae
Kiritimatiellaeota
Lentisphaerae
Nitrospirae
Planctomycetes
Proteobacteria
Spirochaetes
Synergistetes
Thermotogae
Verrucomicrobia
Scaffold length
200000
400000
600000

◂**Fig. 3** Identification of protein-degraders in the bacterial community. The scaffold coverage from the generated metagenome with a minimum length of 5000 bp is plotted against the GC-content, the dot sizes indicate the length of the scaffold. **a**) The scaffolds holding labelled proteins identified by stable isotope probing (SIP) and proteogenomics are highlighted in yellow (LP incubation) and red (LPG incubation), and the affiliated phyla are coloured. **b**) The phyla holding protease according to literature are coloured and the genera are highlighted in white circles

sulphoxide, and trimethylamine N-oxide, or inorganic compounds, *i.e.*, nitrate (NO_3^-), nitrite (NO_2^-), nitrous oxide (N_2O), chlorate (ClO_3^-), perchlorate, oxidised manganese ions, ferric iron (Fe^{3+}), gold (Au), selenate, arsenate, sulphate (SO_4^{2-}) and elemental sulphur (S^0) (Caspi et al. 2018). Possibly, the labelled *Acinetobacter* in the LP and LPG incubations degraded protein through anaerobic respiration, using terminal electron acceptors such as SO_4^{2-}, phosphate (PO_4^{3-}) and Fe^{3+} that are supposed to be present in slaughterhouse wastewaters.

Another possible e-acceptor is oxygen (O_2) that might have been present in the gas collection tube that was connected to the outlet of the lid. However, the total volume of liquid samples taken was 25 mL during the 96 h incubation. Based on this and ignoring the gas production from substrate degradation, a maximum total volume of mL of O_2 (*i.e.*, 7.1 mg COD) could have diffused back from the tube into the incubation bottles due to pressure change. Considering the reduced TCOD of 1000 ± 100 mg·L^{-1} in the LP and LPG incubations, the amount of O_2 was too low to serve as the terminal electron acceptor.

Due to a lack of quantification of the potential terminal electron acceptors, the actual metabolic pathway for anaerobic protein degradation by *Acinetobacter* could not be concluded. Additionally, in retrospect, our study faced a limitation due to the insufficient sequencing depth. This inadequacy resulted in a dataset that lacked the necessary resolution for a thorough and comprehensive analysis. Consequently, the limited data hindered our ability to

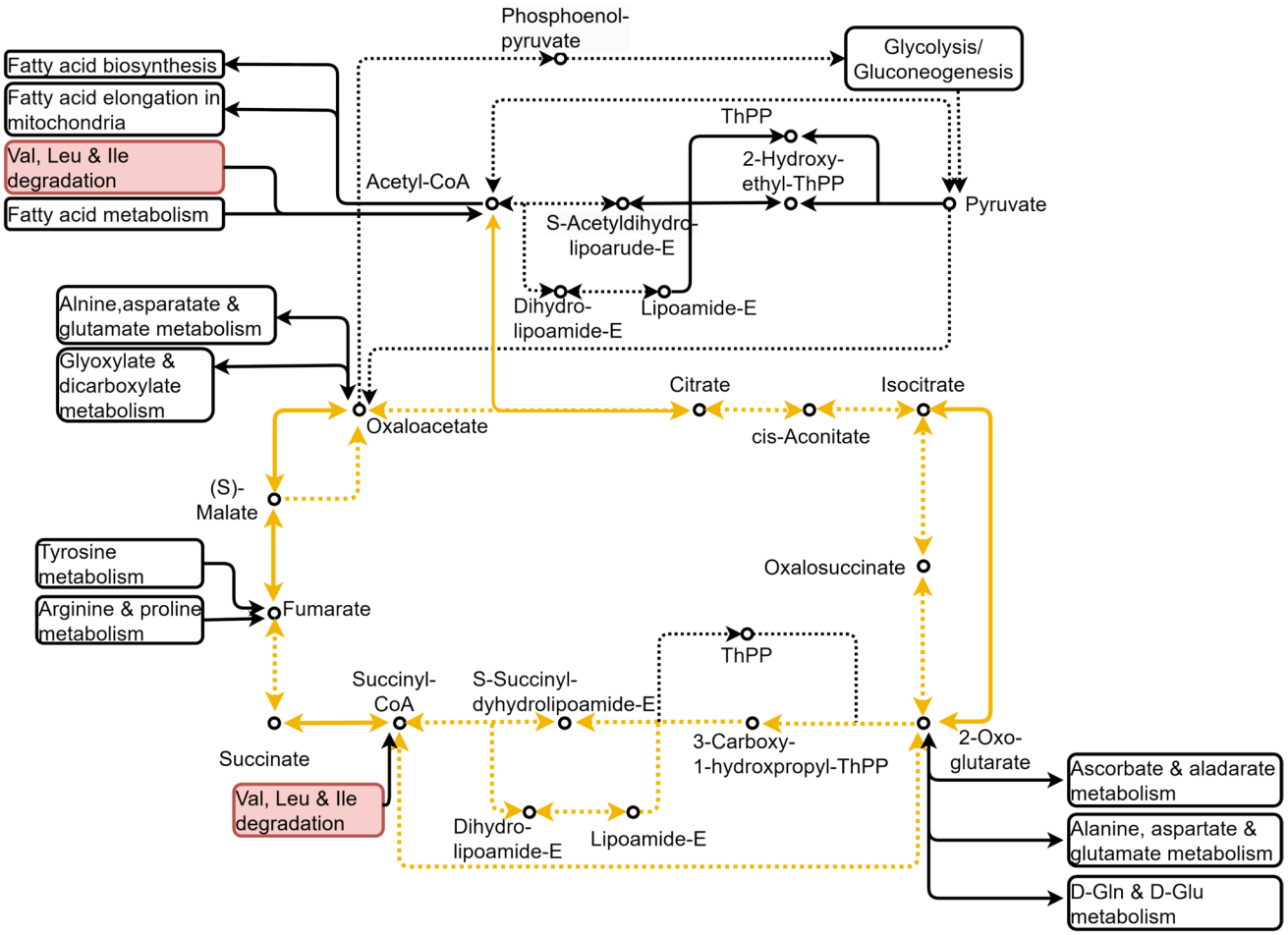

Fig. 4 Model of the valine (Val), leucine (Leu), and isoleucine (Ile) degradation through TCA cycle. The Model was constructed based on the extracted Acinetobacter MAG by using the "Gapfill Metabolic model" in KBase, the TCA cycle is highlighted in yellow, and the presence of metabolic reactions are indicated by solid line

draw robust conclusions and may have overlooked subtle but important details within the metagenome. Future studies should aim for greater sequencing depth to ensure more detailed and accurate insights, as well as examine the anaerobic metabolism of glucose and protein by *Acinetobacter* experimentally.

Implications for AD of protein-rich streams

Although the terminal electron accepter could not be determined, *Acinetobacter* seemed to primarily metabolise proteins and glucose through anaerobic respiration according to the built metabolic model (Fig. 4). Anaerobic respiration of glucose is more energy-efficient than amino acids, *i.e.*, the glycolysis of 1 mol of glucose to pyruvate provides 2 mol ATP (Melkonian and Schury 2019), whereas the conversion of 1 mol of alanine or serine to pyruvate provides 0 mol ATP (Jakubowski et al. 2022). The reason for the lower NH_4^+-N/TN conversion ratio measured in the LPG incubation conducted with a mixture of proteins and glucose can be attributed to a higher energy efficiency in the anaerobic respiration of glucose than amino acids, thus resulting in less proteins degraded and less NH_4^+ produced. Additionally, the relative abundance of *Acinetobacter* in the LPG incubation was 0.5—1.0% lower than in the LP incubation (Fig. 2b), which might also contribute to the lower protein degradation.

Based on the results, we propose implementing a feast-and-famine feeding regime to enhance sequential substrate utilization, prioritizing carbohydrates first, followed by proteins. Previous studies have primarily focused on increasing the C/N ratio to restrict protein degradation and avoid ammonia inhibition. However, this approach also restricts the degradation of total organic carbon, as proteins are part of the total organic matter and contribute to biogas production. A feast-famine scheme allows the depletion of compounds that are rapidly metabolised (e.g., carbohydrates) during the feast phase and the degradation of the slower-biodegradable compounds (e.g., proteins) in the famine phase. Protein accumulation has been observed to be lower in an AnSBR compared to a continuously-fed anaerobic reactor (Tan et al. 2021). Therefore, applying long HRTs or sequential batch feeding to achieve feast-famine scheme is recommended for AD of protein-rich streams. Besides, it is also recommended to increase the population of active protein-degraders within the system, as the enrichment of protein-utilizing genera greatly correlated with higher biogas production and higher methanogens population (Chen et al. 2018).

This proposed approach can optimize energy production and nitrogen recovery without increasing the C/N ratio. By leveraging a mechanistic understanding of microbial behavior, this strategy offers a practical and targeted solution for process optimization in anaerobic digestion systems. It is designed to facilitate protein degradation while safeguarding downstream resource recovery, including energy and ammonia recovery.

Conclusions

In this study, we aimed at the identification of the main bacterial populations that degrade proteins in the environment of an anaerobic digester. From incubations with a ^{13}C-labelled proteinaceous substrate followed by protein-SIP and proteogenomics analyses, we identified the genus *Acinetobacter* as one predominant active degrader of proteins in both low and high concentrations of glucose in the medium. It is the first time that *Acinetobacter* is observed to consume proteinaceous substrate under anaerobic conditions. Outer membrane-bound proteins and porin proteins were the most frequently labelled ones by ^{13}C in the biomass. These proteins can act as cell envelope-bound proteases or peptide transporters. Metabolic pathway analysis conducted on *Acinetobacter* MAG highlighted that amino acids can be anaerobically respired in the presence of alternative electron acceptors, *e.g.*, sulphate and ferric iron, that are likely present in slaughterhouse wastewater. However, the anaerobic metabolism of glucose and proteins by *Acinetobacter* remains to be elucidated experimentally in future research. Additionally, more comprehensive analysis requires in-depth sequencing data. In sum, we elucidated that the retardation on anaerobic protein degradation in the presence of carbohydrates was due to the substrate preference of the main protein-degraders, *Acinetobacter*. We recommended the implementation of a staged or time-phased operational scheme and enrichment of protein-degraders for the effective anaerobic treatment of protein-rich streams.

Abbreviations *AD*: Anaerobic Digestion; *VFA*: Volatile Fatty Acids; *SIP*: Stable Isotope Probing; *AnSBR*: Anaerobic Sequencing Batch Reactor; *TSS*: Total Suspended Solids; *VSS*: Volatile Suspended Solids; *UP*: Unlabelled Protein; *LP*: Labelled Protein; *LPG*: Labelled Protein and Glucose; *TN*: Total Nitrogen; *TCOD*: Total Chemical Oxygen Demand; *SCOD*: Soluble Chemical Oxygen Demand; *C2*: Acetic acid; *C3*: Propionic acid; *C4*: Butyric acid; *iC4*: Iso-Butyric acid; *C5*: Valeric acid; *iC5*: Iso-Valeric acid; *iC6*: Iso-Caproic acid; *OTU*: Operational Taxonomic Unit; *MAG*: Metagenome-Assembled Genomes; *GC-content*: Guanine-Cytosine content

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Author contributions ZD, JLN, and DGW conceived and designed the study concept. ZD designed and conducted the incubation experiment. JSP performed the proteogenomic analysis under the supervision of JLN. ZD and JSP performed the data analysis, interpreted the results, and drafted the manuscript. JLN, DGW, HS, and JBvL supervised the data analysis and interpretation and revised the drafts of the manuscript. All authors contributed to the review and editing of the paper.

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Data availability The amplicon sequences were deposited at DDBJ/EMBL/GenBank under the accession numbers OP474180-OP476210 and the three obtained MAGs were deposited under the accession number SRR30024180-SRR30024182.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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