The metabolic influence of the core ciliate *Entodinium caudatum* within the rumen microbiome

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ABSTRACT

Protozoa comprise a major fraction of the microbial biomass in the rumen microbiome, of which the genus *Entodinium* has been consistently observed to be dominant across a diverse genetic and geographical range of ruminant hosts. Despite the apparent core role that species such as *Entodinium caudatum* exert, their major biological and metabolic contributions to rumen function remain largely undescribed. Here, we have leveraged (meta)genome-centric metaproteomes from rumen fluid samples originating from both cattle and goats fed diets with varying inclusion levels of lipids and starch, to detail the specific metabolic niches that *E. caudatum* occupies in the context of its microbial co-habitants. Initial proteome estimations via total protein counts and label-free quantification highlight that *E. caudatum* comprises an extensive fraction of the total rumen metaproteome. Our analysis also suggested increased microbial predation and volatile fatty acid (VFA) metabolism by *E. caudatum* to occur in high methane-emitting animals, although with no apparent direct metabolic link to methanogenesis. Despite *E. caudatum* having a well-established reputation for digesting starch, it was unexpectedly less detectable in low methane emitting-animals fed high starch diets, which were instead dominated by propionate/succinate-producing bacterial populations suspected of being resistant to predation irrespective of host. Finally, we reaffirmed our abovementioned observations in geographically independent datasets, thus illuminating the substantial metabolic influence that

under-explored eukaryotic populations have in the rumen, with greater implications for both digestion and methane metabolism.

Keywords: Protozoa, *Entodinium caudatum*, metaproteomics, metagenomics, multi-omics, methane, rumen

BACKGROUND

Ruminants operate in symbiosis with their intrinsic rumen microbiome, which is responsible for the degradation of forage into nutrients, in the form of volatile fatty acids (VFAs), supplying ~70% of net energy for the host[1]. The rumen microbiome itself is a complex assemblage of bacterial, fungal, archaeal, viral, and protozoal microorganisms whose intricate composition and function is connected to host productivity traits, such as feed efficiency, milk yield, animal health and greenhouse gas (GHG) emissions[2-5]. Large collaborative research efforts have been made to identify and characterize the core rumen microbiome including creating a publicly available catalogue for cultivated and sequenced genomes[6-8]. In the rumen, bacteria is estimated to constitute 50-90 %, protozoa 10-50 %, fungi 5-10% and archaea less than 4% of the total microbial biomass[9,10]. Due to the difficulties of axenically culturing rumen eukaryotic populations and their complex genomic features that are obstinate to current metagenomic technologies, the reconstruction of the rumen microbiome has been heavily biased towards bacterial and archaeal members, whereas the fungal and protozoal contributions of the rumen currently remain poorly characterized. While anaerobic fungi have a reputable role as fibre degraders in the rumen, only 18 anaerobic gut fungi from herbivores are currently described, with only 11 genomes available[11-13]. Similarly, to date few rumen protozoal genomes are sequenced and publicly available, chief among them, the rumen ciliate protozoa *Entodinium caudatum*[14].

Entodinium represents one of the most dominant genera of rumen protozoa, previously being detected in more than 99% of 592 rumen samples at a mean protozoal relative abundance of ~38% (2015 rumen census: 32 animal species, 35 countries)[15]. While *E. caudatum* has been previously observed to stimulate methane production[16], it is believed that this protozoa lacks hydrogenosomes, and instead encodes mitosomes and iron hydrogenases that may indicate hydrogen production, beneficial for methanogenic endosymbionts[17]. Here, we present a genome-centric metaproteomics analysis of the rumen microbiome from two different host species Holstein dairy cows (*Bos taurus*) and alpine goats (*Capra hircus*) that were fed diets of first-cut grassland hay with a 45:55 forage:concentrate ratio, with concentrates supplemented with either no additional lipid (CTL), or corn oil and cracked-wheat starch grains (COS) [5,18,19]. Moreover, metadata revealed that animals fed COS displayed reduced methane emissions, irrespective of host. To describe how these diets affect digestion and production of methane and VFA's, we sought to investigate changes in function and

composition in the complex rumen microbiome. By using shotgun metagenomic sequencing we recovered in total 244 prokaryote metagenome-assembled genomes (MAGs) that together with selected isolate-derived eukaryote genomes[14,20-25] formed the database for our integrated functional analysis of *E. caudatum*. Despite *E. caudatum* having the genetic ability to degrade plant polysaccharides such as starch and produce hydrogen, our analysis showed contrasting data that suggests *E. caudatum* is less metabolically active in the rumen microbiome of animals fed a starch-rich diet. In such a scenario other starch-degrading and/or propionate and succinate producing bacterial genera, as *Prevotella* and *Fibrobacter* and members of the families *Succinivibrionaceae* and *Aminobacteriaceae* appeared to be more prevalent. In concert, our analysis showed that reduced methane production in both cattle and goats eating feeds supplemented with COS is likely caused via a redirection of hydrogen to succinate and propionate production instead of methanogenesis. Finally, by analysing a secondary, geographically independent dataset, we reaffirmed our primary observations of *E. caudatum* dominance and starch-related metabolism, thus supporting our hypothesis that this protozoal species plays a core role in rumen microbiome function.

METHODS

Animal trial and sample handling

The experimental procedures were approved by the Auvergne-Rhône-Alpes Ethics Committee for Experiments on Animals (France; DGRI agreement APAFIS#3277–2015121411432527 v5) and complied with the European Union Directive 2010/63/EU guidelines. Experiments were performed at the animal experimental facilities of HerbiPôle site de Theix at the Institut National de la Recherche pour l'Agriculture, l'Alimentation l'Environnement (INRAE, Saint-Genès-Champanelle, France) from February to July 2016. Experimental design, animals and diets were previously described by Fougère *et al.*[19] and Martin *et al.*[5]. Briefly, 4 Holstein cows and 4 Alpine goats, all lactating, were enrolled in respectively two 4 x 4 Latin square design trials to study the effects of 4 diets over four 28-d experimental periods. The original study included a control diet and 3 experimental diets with various lipid sources[19]. However, in this work, we only focused on the CTL (grass hay and concentrates containing no addition lipids) and COS (CTL diet supplemented with corn oil and wheat starch) diets which were associated with the most extreme methane (CH_4) emission profiles in both ruminant species. In the present study, we focused on the two following diets: a diet composed of grass hay ad libitum with concentrates containing no additional lipid (CTL) or corn oil (5.0% total dry matter intake (DMI)) and cracked-wheat starch -5.0 % of total DMI (COS) (**Table 1**). Corn oil (Olvea, Saint Léonard, France) was added to the concentrate, at 5% of total DMI and contained (g/kg of total FA): 16:0 (114), 18:0 (16.4), cis-9 18:1 (297), cis-11 18:1 (6.30), 18:2n-6 (535), 18:3n-3 (7.57), 20:0 (3.48), 22:0 (1.0),

24:0 (1.5), and total FA (1000 g/kg). Detailed diet composition is available in Martin *et al.*[5]. Each experimental period lasted for 28 days. Diets were offered as 2 equal meals at 0830 and 1600h. Animals had access to a constant supply of freshwater ad libitum. Concentrate and hay refusals were weighed daily. The amounts of feed offered the following day was adjusted regarding to refusals to maintain the targeted the dietary 45 % dry matter (DM) forage and 55 % DM concentrate ratio.

Table 1. Summary of the animal host, dietary conditions and accompanying metadata that are linked to the rumen samples used to explore *E. caudatum* function. Effects of control diet (CTL) and diet supplemented with corn oil and wheat starch (COS) on average VFA concentration in the percentage of total VFA concentration (mmol/l) and average methane yield (CH_4 g/kg DMI) in addition to average total protozoa cell counts and average cell counts for small *Entodiniomorph* protozoa for cows (n=4) and goats (n=4) (**Supplementary Table S1**)[5]. Total protozoal cell counts, and cell counts of small *Entodiniomorph* protozoa were based on ciliate protozoa morphology in microscopy[5]. Measurements on VFA concentrations and methane yield were determined by gas chromatography and respiration chambers, respectively.

Animal	Diet	VFA sum mmol/l	Acetate (% sum)	Propionate (% sum)	Butyrate (% sum)	CH_4 g/kg dry matter intake	Standard deviation	Total protozoa (10^3 cells/ml)	Small entodiniomorphs (<100µm) (10^3 cells/ml)
COW	CTL	61.83	72.02	14.21	10.80	19.51	2.18	92	51
	COS	68.06	69.12	21.32	6.39	14.45	2.89	100	97
GOAT	CTL	33.70	65.73	15.62	13.66	19.52	3.96	1382	1346
	COS	25.27	64.17	19.07	9.90	13.47	4.14	898	898

Rumen fluid was collected through stomach-tubing before the morning feeding on day 27 of each experimental period. The stomach tube consisted of a flexible 23 mm external diameter PVC hose fitted to a 10 cm-strainer at the head of the probe for cows, and a flexible 15 mm PVC hose with a 12 cm-strainer for goats. The first 200 ml of rumen fluid was discarded from to minimize contamination from saliva. Samples were filtered through a polyester monofilament fabric (280 µm pore size), dispatched in 2-ml screw-cap tubes, centrifuged at 15000 x g for 10 mins and the pellet snap-frozen in liquid nitrogen. Samples were stored at -80°C until DNA extraction using the Yu and Morrison bead-beating procedure [26]. In total, 32 rumen fluid samples (4 cattle and 4 goats fed four diets included in the original study[19]) were sent to the Norwegian University of Life Sciences (NMBU) for metagenomic and metaproteomic analysis. Respiration chambers were used to measure methane emissions over a 5-day period, while VFA and NH_3 concentrations were determined by gas chromatography using a flame ionization detector[27]. Protozoa were counted by microscopy and categorized as either small entodiniomorphs (<100 µm), large entodiniomorphs (>100 µm) or as holotrichs *Dasytricha* and *Isotricha*[9]. Further specifics about diets and measurements can be found in Martin *et al.*[5] and VFA and methane measurements are summarized in **Table 1**.

Metagenomic sequencing and analysis

Metagenomic shotgun sequencing was performed at the Norwegian Sequencing Centre on two lanes of the Illumina HiSeq 3/4000 generating 150 bp paired-end reads in both lanes. Sequencing libraries were prepared using the TruSeq DNA PCR-Free High Throughput Library Prep Kit (Illumina, Inc) prior to sequencing. All 32 samples (4 cattle and 4 goats fed four diets included in the original study[19]) were run on both lanes to prevent potential lane-to-lane sequencing bias. FASTQ files were quality filtered and Illumina adapters removed using Trimmomatic [28] (v. 0.36) with parameters -phred33 for base quality encoding, leading and trailing base threshold set to 20. Sequences with an average quality score below 15 in a 4-base sliding window were trimmed and the minimum length of reads was set to 36 bp. MEGAHIT [29] (v.1.2.9) was used to co-assemble reads originating from samples collected from cow and goats separately, with options –kmin-1pass, --k-list 27,37,47,57,67,77,87, --min-contig-len 1000 in accordance with [7]. Bowtie2[30] (v. 2.3.4.1) was used to map reads back to the assemblies and SAMtools[31] (v. 1.3.1) was used to convert SAM files to BAM format and index sorted BAM files.

The two co-assemblies (one from the samples originating from cattle and the other originating from the samples of goats) were binned using Maxbin2[32], MetaBAT2[33] and CONCOCT[34]. MetaBAT2 (v. 2.12.1) was run using parameters –minContig 2000 and –numThreads 4, Maxbin2 (v. 2.2.7) ran with default parameters and -thread 4, min_contig_length 2000, and CONCOCT (v. 1.1.0) ran with default parameters and –length_threshold 2000. Further, bins were filtered, dereplicated and aggregated using DASTool[35](v. 1.1.2) with the parameters –write_bins 1, --threads 2 and BLAST[36] as search engine. This resulted in a total of 244 dereplicated MAGs across the two host species (104 originating from cow and 140 from goat). CheckM[39](v. 1.1.3) lineage workflow was used to determine completeness and contamination of each MAG, with parameters –threads 8, --extension fa, and CoverM (v. 0.5.0) (https://github.com/wwood/CoverM) was used to calculate relative abundance of each MAG, while GTDB-tk[40,41] (v. 1.3.0) was used for taxonomic annotation. 90% (219 of 244) of the recovered MAGs were considered high or medium quality MAGs according to MIMAGs threshold for completeness and contamination for genome reporting standards[42]. Gene calling and functional annotation of the final MAGs were performed using the DRAM[43] pipeline with the databases dbCAN[44], Pfam[45], Uniref90[46], Merops[47], VOGdb and KOfam[48]. The translated amino acid sequences from the publicly available drafted *E. caudatum* macronucleus genome were annotated with the KEGG metabolic pathway database using BlastKOALA[49] by Park *et al.* [14]. Proteins with resulting KEGG Orthology (KO) numbers were functionally assigned to metabolic pathways using KEGG Mapper Reconstruct Tool [50].

The resulting protein sequences for each MAG, as well as those from the host genomes of goat (*Capra hircus*, NCBI ID: 10731) and cattle (*Bos taurus*, NCBI ID: 82) were compiled into two databases, from now on referred to as sample specific RUmen DataBase for Goat (RUDB-G) and sample specific RUmen DataBase for cattle (RUDB-C). In addition, the genomes of the protozoa *Entodinium caudatum* [14] and *Fibrobacter succinogenes* S85 (NCBI ID: 932) was added to both rumen databases. *F. succinogenes* is well recognized as a primary cellulolytic bacterium in the rumen microbiome and has previously been observed as an active microorganism in similar studies yet was not thoroughly binned as a unique MAG in this study. Nine available fungal genomes downloaded from Joint Genome Institute (JGI) Mycocosm[51] were added to RUDB-C (*Anaeromyces* sp. S4[21], *Caecomyces churrovis*[24], *Neocallimastix californiae*[21], *Neocallimastix lanati*[25], *Piromyces finnis*[21], *Piromyces* sp. E2[21], *Piromyces* UH3-1[20,21,23], *Piromyces* eukMAG[52], *Orpinomyces* sp.[22]). Because of database size restrictions in downstream analysis of metaproteomics data[53], only four of the nine fungal genomes were added to RUDB-G (*Anaeromyces* sp. S4, *Caecomyces churrovis, Neocallimastix lanati, Piromyces* UH3-1). In total the complete databases consisted of 452 073 and 431 023 protein entries for RUDB-G and RUDB-C, respectively.

Metaproteomic data generation

To 300 µL of rumen fluid sample (in total 32 rumen fluid samples originating 4 cattle and 4 goats fed four diets included in the original study[19]) 150 µL lysis buffer (30 mM DTT, 150 mM Tris-HCl (pH=8), 0.3% Triton X-100, 12% SDS) was added together with 4 mm glass beads (≤ 160 µm), followed by brief vortexing and resting on ice for 30 mins. Lysis was performed using FastPrep-24™ Classic Grinder (MP Biomedical, Ohio, USA) for 3 × 60 seconds at 4.0 meter/ second[54]. Samples were centrifuged at 16 000 × *g* for 15 minutes at 4°C and lysate was carefully removed. Protein concentration was measured using the Bio-Rad DC™ Protein Assay (Bio-Rad, California USA) with bovine serum albumin as standard. Absorbance of sample lysates was measured at A750 on BioTek™ Synergy™ H4 Hybrid Microplate Reader (Thermo Fisher Scientific Inc., Massaschusetts, USA). 40-50 µg of protein was prepared in SDS-buffer, heated in water bath for 5 minutes at 99°C and analysed by SDS-PAGE using Any-kD Mini-PROTEAN TGX Stain-Free™ gels (Bio-Rad, California, USA) in a 2-minute run for sample clean-up purposes, before staining with Coomassie Blue R-250. The visible bands were carefully excised from the gel and divided as 1×1 mm pieces before reduction, alkylation, and digestion with trypsin. Peptides were concentrated and eluted using C18 ZipTips (Merck Millipore, Darmstadt, Germany) according to manufacturer's instructions, dried, and analysed by nano-LC-MS/MS using a Q-Exactive hybrid quadrapole Orbitrap MS (Thermo Fisher Scientific Inc., Massaschusetts, USA) as previously described[55]

Metaproteomic data analysis

Acquired MS raw data were analysed using MaxQuant[56] (v. 1.6.17.0) and searched against the RUDB's. The MaxLFQ algorithm was used to quantify proteins[57]. Detected protein groups were explored in Perseus[58] (v. 1.6.8.0). Both RUDB's were supplemented with contaminant protein entries, such as human keratin, trypsin, and bovine serum albumin, in addition to reversed sequences of all protein entries for estimation of false discovery rates (FDR). Oxidation of methionine, protein N-terminal acetylation, deamination of asparagine and glutamine, and conversion of glutamine to pyroglutamic acids were used as variable modifications, while carbomidomethylation of cysteine residues was used as fixed modification. Trypsin was chosen as digestive enzyme and maximum missed cleavages allowed was one. Protein groups identified as potential contaminants were removed. Proteins were filtered to 1% FDR and considered valid if they had at least one unique peptide per protein and at least one valid value in total. One sample originating from goat fed HPO diet (14201 P2 (Goat 2 fed HPO P2, sample no. 22)) was deemed as a technical outlier, mapping significantly fewer protein groups in metaproteomic analysis compared to the rest of the data and was therefore removed from the downstream analysis. After filtration, we resolved 1081 unique protein groups across the 16 samples from cattle and 1632 unique protein groups across 15 samples from goats. Box plots for **Figure 1** were made with ggplot2[59] in R (v. 4.2.0)[60]. To determine which expressed metabolic pathways *E. caudatum* were significantly enriched for in each diet/animal, we used the hyperR package[61] in R which employs the hypergeometric test. The 'geneset' for hyperR was generated by using the KEGGREST R package to retrieve entries from the KEGG database and determine which pathways the *E. caudatum* KOs belong to. The geneset was then manually curated to only include metabolic pathways of interest (i.e., we remove pathways such as "Huntington disease"). For the 'background' setting in hyperR, to be conservative, we used the total number of unique KOs (7592) in the *E. caudatum* genome that could possibly be expressed.

Animal trial, sample handling and metagenomic data generation for independent validating dataset

Samples were also analysed from previously performed feeding experiments with Holstein Friesian bulls[62]. In brief, these bulls were subjected to either *ad libitum* or restricted feeding regime in a compensatory growth model detailed in Keogh *et al.*, 2015[62]. Both feeding groups received the same ratio of concentrate and grass silage, respectively 70% and 30%, of which the concentrate was mainly composed of starch-rich rolled barley (72.5%) and soya (22.5%). Rumen samples were collected at slaughter and stored at -80C prior to metagenomics and metaproteomic analysis in this study.

Sample preparation, cell lysis and extraction of DNA was carried out as previously described by McCabe *et al.*[63] Quality check of fastq files and removal of low-quality reads was performed using fastp (V.0.19.5). Sequence reads were mapped against the bovine genome (ARS-UCD1.3) using minimap2 (V.2.16), and host sequences were removed. Reads were co-assembled using Megahit (V1.2.6) with "–meta-large" pre-set option as the metagenome was complex. Metagenomic binning was applied to the co-assembly using MetaBAT2 using standard parameters (V.2.12.1). MAGs were then dereplicated using dRep (V.1.4.3), and the resulting MAGs were taxonomically annotation using Bin Annotation Tool (BAT), available on (https://github.com/dutilh/CAT). This tool internally uses prodigal (V.2.6.3) for gene prediction and DIAMOND (V.0.9.14) for the alignment against the non-redundant (nr) protein database (As of Feb 2020).

Sample preparation for metaproteomics was done by lysing cells with bead beating with two glass bead sizes (≤106 µm and 0.5 mm), in 100 mM Tris, pH8, 5% SDS and 10 mM DTT. A FastPrep 24 instrument was operated for 3 × 45 seconds at a speed of 6.5 m/s. The samples were centrifuged for 15 minutes at 20.000 × *g* and the protein extracts were cleaned by Wessel-Flügge precipitation[64]; pellets were dissolved in 5% SDS, 100 mM Tris-Cl, pH8, 10 mM DTT and kept at -20 °C until further processing. Protein digestion was performed using suspension trapping (STrap)[65], dried in a SpeedVac (Eppendorf Concentrator Plus) and re-dissolved in 0.05 % trifluoroacetic acid, 2% acetonitrile for peptide concentration estimation using a Nanodrop One instrument, and subsequent MS/MS-analysis. The samples were analyzed using an Ultimate3000 RSLCnano UHPLC coupled to a QExactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Fisher, Bremen, Germany) as described previously[55].

Mass spectrometry raw data were analysed with a sequence of software in the Galaxy software suite (usegalaxy.eu). Initially, they were searched against the sample-specific protein sequence database (1.773.447 protein sequences) with SearchGUI[66] utilizing the X!Tandem search engine[67] version Vengeance. The database was supplemented with contaminant protein entries, such as human keratin, trypsin, and bovine serum albumin, in addition to reversed sequences of all protein entries for estimation of false discovery rates (FDR). Oxidation of methionine and protein N-terminal acetylation were used as variable modifications, while carbomidomethylation of cysteine residues were used as fixed modification. Trypsin was chosen as digestive enzyme, maximum missed cleavages allowed was one and matching tolerance levels for MS and MS/MS were 10 ppm and 20 ppm, respectively. PeptideShaker[68] was used to filter the results to 1% FDR and quantification was done using FlashLFQ[69] including normalization between samples and the feature 'match between runs' to maximize protein identifications. Perseus[58] version 1.6.2.3 was used for further analysis. A protein

group was considered valid if it had at least one unique peptide identified and being quantified in at least 50% of the replicates in at least one condition (7 restricted and 8 Ad lib). Protein groups identified as potential contaminants were removed. Calculations of MAG abundances were done by summing LFQ values for all proteins belonging to each MAG and differential abundance between diets were detected by a two-sided Student's t-test ($p<0.05$).

RESULTS AND DISCUSSION

Protozoal populations have large proteomes in the rumen microbiome

Because of their large size protozoal species can comprise a significant fraction of the microbial biomass in the rumen[9]. While the total number and diversity of protozoal species are lesser than their bacterial counterparts in the rumen, their genome size and total gene count are considerably larger and due to alternative splicing and post-translational modifications, the protein representation of protozoal populations will be larger than the number of genes in the genome[70]. Thus, the amount of protein in a protozoa species can be expected to far exceed the amount of protein in a bacterial species that can be identified and quantified in proteomic studies. In this context, our metaproteomic data showed an extensive fraction of detectable proteins affiliated to *E. caudatum*, and other closely related species, in both cows (26.3%) and goats (31.5%) in proportion to the combined bacterial species that were represented in our genome databases (**Figure 1a**). In addition, the label free quantification (LFQ) of *E. caudatum* proteins, which is indicative of protein detection intensity, was proportionally higher than the bacterial fraction of the rumen microbiome further supporting the dominance of protozoal activity in our samples (**Figure 1b**). Twice as many *E. caudatum* proteins were detected in goat than cows (mean: 514 vs 284), however this was somewhat expected given the 7x higher counts of entodiniomorph concentration (cells/mL) previously observed in the goat samples compared to cows (**Table 1**)[5].

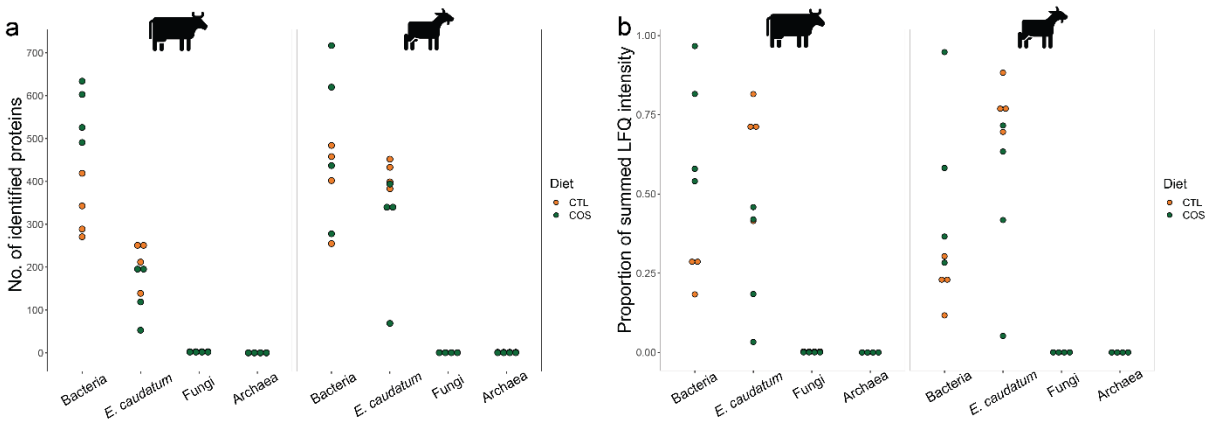

Figure 1. Quantities of proteins identified as *E. caudatum* in the rumen microbiome vary depending on host animal and dietary conditions. Boxplots for total proteins identified (**a**), and average recovered metaproteomic expression (**b:** presented as proportion of summed LFQ intensities) belonging to protozoal (*E. caudatum*), bacterial, archaeal, or fungal species across the control diet (CTL) and diets supplemented with corn oil and

wheat starch (COS) for dairy cows (n=4) and goats (n=4). Detected proteins LFQ intensities for protozoal and bacterial populations can be found in **Supplementary Table S2**.

Metabolism of E. caudatum shows predatory activity and metabolism of VFAs

While previous efforts have investigated the genome and transcriptome of *E. caudatum* grown in monoculture[14,71], our metaproteomic analysis sought to reveal *in vivo* metabolism and functions of *E. caudatum* within the rumen microbiome. In accordance with Wang *et al.*[71], our metaproteomic analysis revealed expressed proteins significantly enriched in metabolic pathways such as carbon metabolism, glycolysis/gluconeogenesis, starch and sucrose (and glycogen) metabolism, pyruvate metabolism, oxidative phosphorylation and production of alcohol (**Supplementary Table S3**). Wang *et al.* found that as for most rumen microbes, *E. caudatum* uses carbohydrates such as starch as its primary substrate, as well as cellulose and hemicellulose to a certain degree[71], and their transcript analyses showed that *E. caudatum* had high levels of expression of amylases and low-level expression of hemicellulases, cellulases and pectinases. Similarly, our metaproteomic analysis reveals expression of amylases by *E. caudatum* that are predicted to enable *E. caudatum* to engulf and degrade starch granules to simpler sugars and to produce glycogen, its most important storage carbohydrate[72]. However, no detection of *E. caudatum* carbohydrate active enzymes (CAZymes) related to hemicellulose or pectin were observed in any of our metaproteomes, suggesting that it is not engaging in the deconstruction of these carbohydrates at the time our samples were collected for analysis (before feeding). It should be noted that ruminal fermentation activity as well as production of VFA's and methane will be at its highest after feeding, as a result of an increased availability of fermentable substrate[73]. While sampling time can influence the recovered microbial composition and hence function, any differences in metabolic parameters or species abundance in this study is relative across both diets given the consistent sampling times.

While monoculture cultures of *E. caudatum* have not been established to verify the VFA's it can produce, Wang *et al.* found transcripts of enzymes involved in fermentative formation of acetate and butyrate[71]. Similarly, we detected proteins inferred in metabolism of acetate, butyrate, and alcohol in *E. caudatum*. Irrespective of host, animals fed the control (CTL) diet had a higher proportion of *E. caudatum* proteins and concurrently had increased relative levels of acetate and butyrate compared to animals fed the corn oil and wheat starch diet (COS), which had fewer *E. caudatum* proteins and lower acetate/butyrate levels (**Figure 1** and **Table 1**). As *E. caudatum* was seemingly most abundant in goats fed the CTL diet, we used these metaproteomes to reconstruct metabolic features (**Figure 2**). Of the 514 *E. caudatum* proteins identified in goats, 454 had unique KO numbers assigned, from which KEGG Mapper reconstructions[50] enabled functional assignment of 268 proteins to metabolic

pathways. Our metabolic reconstructions showed expressed proteins involved in endocytosis, phagosome and lysosome processes for predatory activity, engulfment, and digestion of bacteria (**Supplementary Table S2**). Interestingly, for the rumen samples used in this study Martin *et al.* previously observed higher NH_3 concentrations in goats compared to cows[5] and hypothesised that it might have resulted from increased bacterial protein breakdown and feed protein degradability due to higher density of entodiniomorphs known for their predatory activity[5]. In support of these observations, we performed metaproteomic pathway enrichment analysis of *E. caudatum* (**Figure 2, Supplementary Table S3**), which revealed significantly enriched nitrogen metabolism, in addition to purine and pyridine metabolism in goats but not in cows. Other biological processes such as signalling and metabolism of amino acids, and amino and nucleotide sugars represented significantly enriched pathways in *E. caudatum* (**Supplementary Table S3**). In the previous transcriptome study by Wang *et al.*, transcripts for a ferredoxin hydrolase and an iron hydrogenase were recovered and are suspected to be involved in production of hydrogen. Here in our metaproteomic analysis, we identified only one of its eight iron hydrogenases (in goats, it was absent in cows, **Supplementary Table S2**), which showed no contrasting changes in LFQ intensity in either the CTL or COS datasets, contributing to the uncertainty that *E. caudatum* is a major producer of hydrogen, despite previous reports associating its abundance with higher methane levels [74-76].

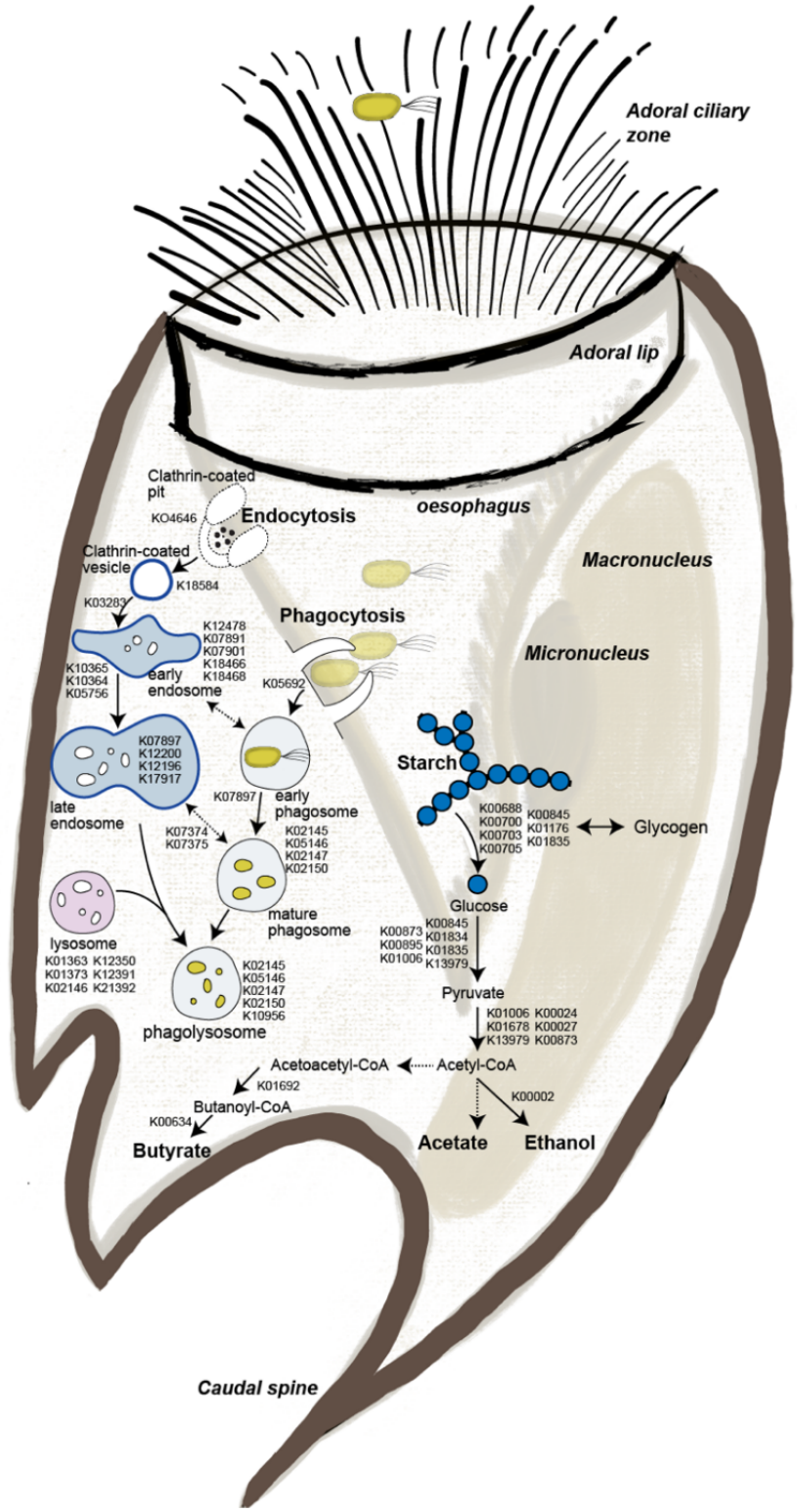

Figure 2. Reconstructed phagolysosome formation and starch metabolism of *Entodinium caudatum* within the rumen microbiome of goats fed the control diet (CTL) based on metaproteomic analysis. KO identifiers for identified proteins were analysed via KEGG mapper to reconstruct expressed key features in the metabolism of *E. caudatum*. Dashed arrows represent proteins or pathways that were not detected in our metaproteomes but are key steps in their respective pathways. Detailed information connecting KO identifiers to their respective gene ID, LFQ and animal/diet can be found in **Supplementary Table S2**.

E. caudatum is less active in diets supplemented with starch regardless of its starch degrading reputation.

The changes in VFA and methane levels in animals fed the high starch COS diet, previously measured by Martin *et al.*[5], suggested significant alterations in composition and thus functions of the rumen microbiome irrespective of host species. In particular, a decrease in proportions of acetate and butyrate, decrease in the acetate:propionate ratio and an increase in proportional propionate levels were observed in animals fed the COS diet, compared to the CTL diet (**Table 1**). Diets that are high in starch content or with low forage:concentrate ratios have previously been shown to result in higher production of propionate and succinate, as they are easily fermented in the rumen and accordingly have high passage rates[77,78]. We therefore leveraged our genome-centric metaproteomic data from both cows (**Figure 3a-c)** and goats (**Figure 3d-f**) fed either the COS or CTL diet to gain an overview of protein expression from individual populations. We specifically focused on pathways involved in the degradation of starch (CTL: corn starch, COS: corn + wheat starch) to pyruvate through glycolysis and finally formation of acetate, butyrate, and propionate (via succinate). Irrespective of host, and despite its starch-degrading reputation[71,72], *E. caudatum* had a lower abundance and less proteins involved in starch degradation in animals fed the COS diet compared to those fed the CTL diet (**Figure 3a** and **3d**). Further, we observed opposing patterns for *E. caudatum* proteins involved in glycolysis, and production of pyruvate, acetate, and butyrate, which were detected in higher levels in both cows and goats fed the CTL diet compared to the starch and corn oil (COS) supplemented diet.

While several putative *E. caudatum* amylases were detected across all animals and diets, their quantification levels (i.e., LFQ intensities) did not increase as expected when higher levels of starch were available (**Figure 3a** and **d**). We therefore hypothesized that the observed shift in VFA profiles in response to increased starch was additionally influenced by the bacterial fraction of the rumen microbiome. In contrast to lower *E. caudatum* levels in the animals fed the COS diet, we observed an increase in suspected starch-degrading bacterial species, and succinate- and propionate-producing bacterial species irrespective of host (**Figure 3c** and **3f**). For example, starch fermentation pathways from population genomes affiliated with the *Succinivibrionaceae* family, *Prevotella* species, *Fibrobacter* species and, additionally for goats, members of the *Selenomonadaceae* and *Aminobacteriaceae* families, were detected at higher proteomic levels in the animals fed the COS diet compared to those fed the CTL diet (**Figure 3c** and **3f**).

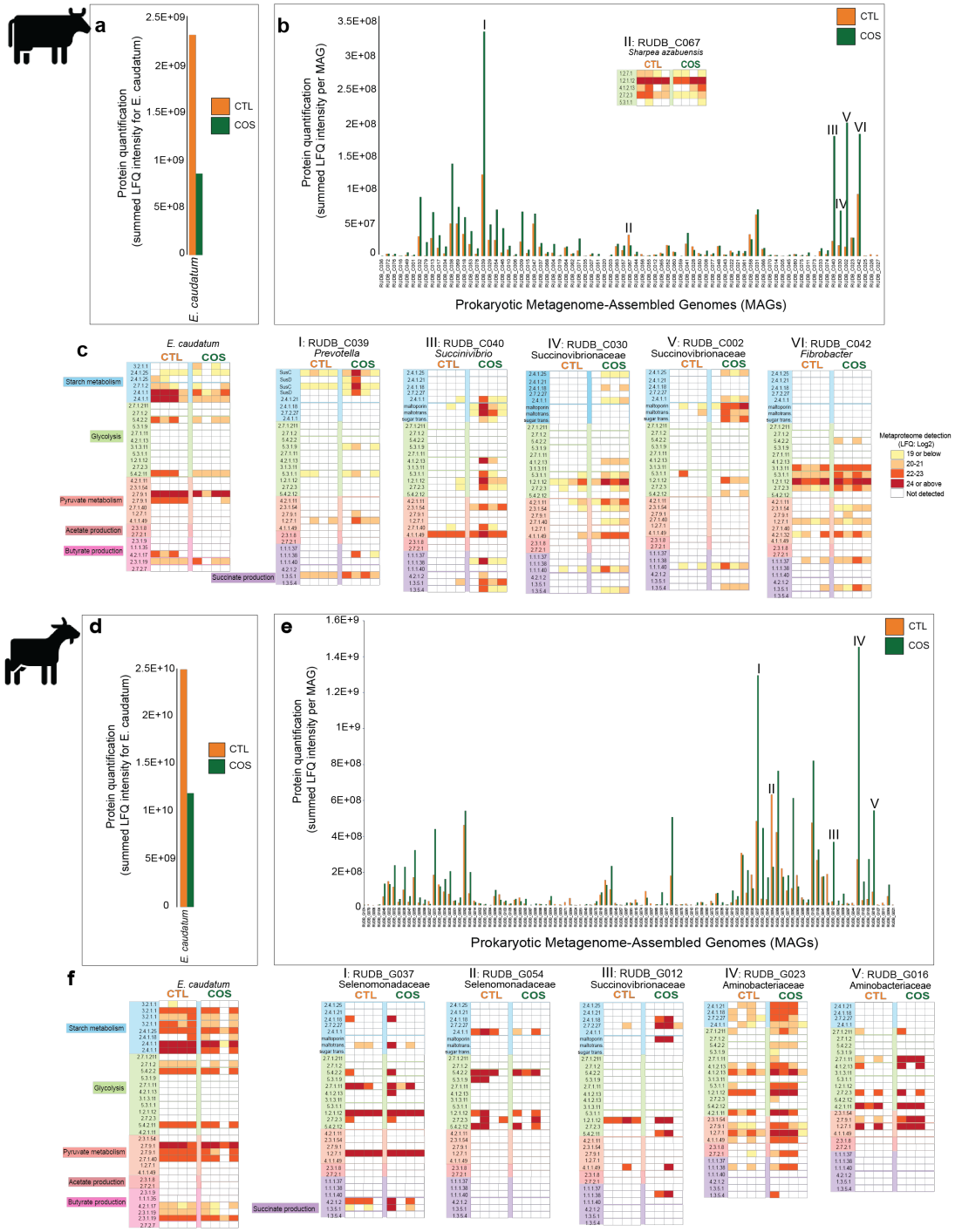

Figure 3. Detected proteins mapped to the genome of *E. caudatum* and bacterial metagenome-assembled genomes (MAGs) in the rumen microbiome of dairy cattle (n=4) and goats (n=4) fed either a control diet (CTL) or one supplemented with corn oil and wheat starch (COS). The figure displays metabolically active populations (as genomes or MAGs), with selected expressed proteins (presented as Enzyme Commission (EC) number) active in starch degradation, glycolysis and production of pyruvate, butyrate, acetate, and succinate in cows (**a-c**) and goats (**d-f**) fed CTL or COS diets. Panels **a** and **d** depict *E.caudatum* proteomes that were detected in cattle and goats respectively are presented separately to bacteria (panels **b** and **e**) as the scale of their protein quantification values were ~10x larger. Protein quantification values (y-axis) were calculated by considering both the number of proteins detected per MAG/genome and their LFQ intensity: we averaged LFQ intensities for each detected protein across biological replicates for each dietary condition (CTL: green or COS: orange), which were subsequently summed for all detected proteins per MAG/genome. Panels **c** and **f** show selected MAGs (I-VI) with metabolically active proteins, presented as EC numbers, recovered from cows (RUDB-C) and goats (RUDB-G) fed

either CTL or COS diets. MAGs are presented with their MAG ID and taxonomic annotation from GTDB-tk. Genome annotations and LFQ intensities used to create panels **a-f** can be found in **Supplementary Table S2**.

E. caudatum is less active in animals that produce lower methane yield

For the animals sampled in this study, Martin *et al.* demonstrated a ~25-30% reduction in methane emissions in both cows and goats fed the COS diet compared to the control (**Table 1**)[5]. While our proteomic evidence clearly showed a lower *E. caudatum* activity in COS-fed low-methane producing animals, a specific mechanism that explains this phenomenon is still elusive. Previous comparisons between defaunated and faunated animals have shown decrease in methane production in protozoa-free ruminants, suggesting symbiotic interactions between methanogenic archaea and protozoal species[74]. Methanogen's epi- and endo-symbiotic relationships with protozoa have also been suggested to contribute to 9-37% of rumen methanogenesis[74,75,79,80]. Moreover, studying microcosms with the presence and absence of protozoal species Solomon *et al.* reported higher levels of acetate and butyrate in microcosms with protozoa present in addition to increased methane emissions[74], which supports the main findings of animals fed the CTL diet in this study (**Figure 4**). It is tempting to speculate that such protozoal-methanogen relationships in this study are centred on hydrogen transfer. However, we observed minimal evidence in our proteomic data that *E. caudatum* makes major contributions to ruminal hydrogen production that is linked to methane levels, with only one of its eight iron hydrogenases detected in goats (absent in cattle), which showed no changes in LFQ intensity in either the high (CTL) or low (COS) methane yielding animals.

Increases in dietary starch for ruminants is known to stimulate the propionate and succinate pathways of starch-degrading bacteria, which due to their net incorporation of metabolic hydrogen [H] represent a [H] sink in rumen fermentation besides hydrogenotrophic methanogenesis[79,81]. In addition to starch, in a study conducted by Zhang *et al.*[82], goats fed corn oil as a supplement decreased ruminal H_2 concentrations and total methane emissions. Nevertheless, there was seemingly no effect on rumen protozoal populations, which suggests that corn oil does not act as an anti-protozoal agent, with the dose of corn oil used in this study[82]. Furthermore, supplementation of dietary lipids can decrease plant fibre degradation and hence levels of acetate and butyrate at the expense of propionate production, as lipid-derived long-chain fatty acids can be toxic to keystone fibre degrading gram-positive bacterial species[83,84]. These findings were in agreement with the decreased CH_4 production in cows and goats in this study fed the COS diet, which was observed to additionally impact other ruminal fermentation parameters, such as increased propionate and decreased butyrate and acetate levels (**Figure 4**)[5].

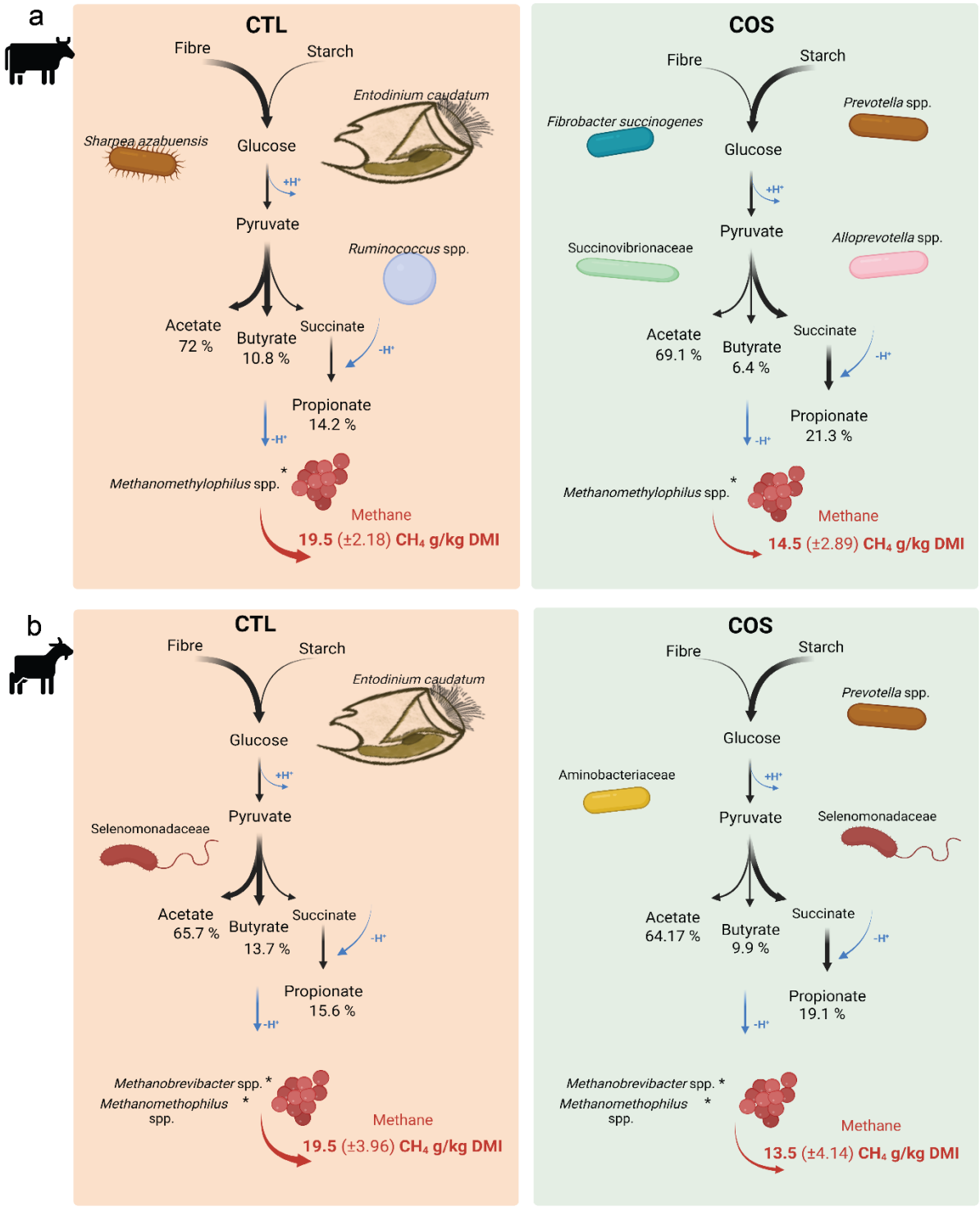

Figure 4. Schematic overview of the overall predicted metabolism in the rumen microbiome of cows (Panel a) and goats (Panel b) fed the control diet (CTL) compared to those fed the diet supplemented with corn oil and wheat starch (COS). The dominant microorganisms, as determined via MAG-centric metaproteomics, were predicted to convert starch into volatile fatty acids (VFAs) and methane in the rumen microbiome. However, the taxonomic and VFA profile of the rumen microbiomes differed when their host animals were subjected to different levels of starch in their diet. Thicker arrows represent higher abundance of metabolites. VFA concentrations were determined using gas chromatography. Percentages of VFA concentrations can be found in Supplementary Table S1. Percentages of VFA concentrations and methane yield are presented at average values, and numbers in parentheses are standard deviation for measured values for methane yield from each diet. * Represented by few detected proteins in the metaproteomic analysis.

Diets rich in starch are more fermentable in the rumen, which can decrease the ruminal pH to levels that can inhibit methanogenic archaea and fibre-degrading bacterial species[85,86]. Yet, lowered pH levels in the rumen can also lead to clinical (or sub-clinical in most production scenarios) ruminal acidosis[87,88]. Hence, high concentrate diets, which increase production of propionate at the expense of methane, does not necessarily opt for a viable methane mitigation strategy in the long term. Our results suggests that decreased methanogenesis in COS-fed animals is likely due to a decrease in available hydrogen and/or decrease in pH levels, which we predict is caused by the metabolism of dominant wheat starch-degrading populations that likely do not produce exogenous hydrogen due to their own [H]-utilizing succinate and propionate metabolism (**Figure 4**).

***E. caudatum* dominance is validated in geographically independent datasets**

To further test our hypothesis that *E. caudatum* plays a central role in the rumen ecosystem, we explored additional metagenome-centric metaproteomic datasets originating from an independent feeding experiment performed in Ireland on 60 Holstein Friesian bulls[62]. In brief, these bulls were subjected to the same ratio of concentrate and grass silage at either an *ad libitum* or restricted feeding regime in a compensatory growth model detailed in Keogh et al, 2015[62]. We applied the same strategy as for the described Holstein dairy cows and alpine goats to resolve the metaproteomic dataset for a subset of 15 animals (7 restricted and 8 Ad libitum) against 781 reconstructed sample-specific MAGs (RUDB-HF), which were supplemented with the genome of *E. caudatum,* as well as genomes of available anaerobic fungi. This collection of microbial prokaryote and eukaryote genomes was then used as a sequence database for the generated protein spectra. Consistent with our previous observation, a substantial proportion of the detected proteins were affiliated to *E. caudatum* providing further support that *Entodinium* is an important and metabolically active contributor to the rumen microbiome. Intriguingly, the protein quantification (measured as sum of LFQ intensities affiliated to each MAG/genome, averaged for each diet) was twice as high in the rumen sample from bulls on the restricted diet, which likely had less starch available compared to the *ad libitium* group and a higher retention time (**Figure 5a**). A previously published 16S rRNA amplicon investigation of the phylogenetic differences between the rumen microbiomes of these two diet groups highlighted an increase in *Succinivibrionaceae* in the starch-rich *ad libitium* diet[63]. Our metaproteomic analysis confirmed a significantly higher ($p < 0.05$) proteomic detection of several *Succinivibrionaceae*-MAGs under the *ad libitium* group (**Figure 5b**), accompanied with a reduced acetate:propionate ratio in the rumen, which is often associated with increased feed efficiency and reduced production of methane[63]. These observations largely mirror the dominance of *Succinivibrionaceae*-MAGs in the dairy cattle and

goats fed the COS diet, further strengthening our hypothesis that *E. caudatum* does not metabolically respond to increases in available starch in the host animals' diets and has other roles than being a primary starch degrader.

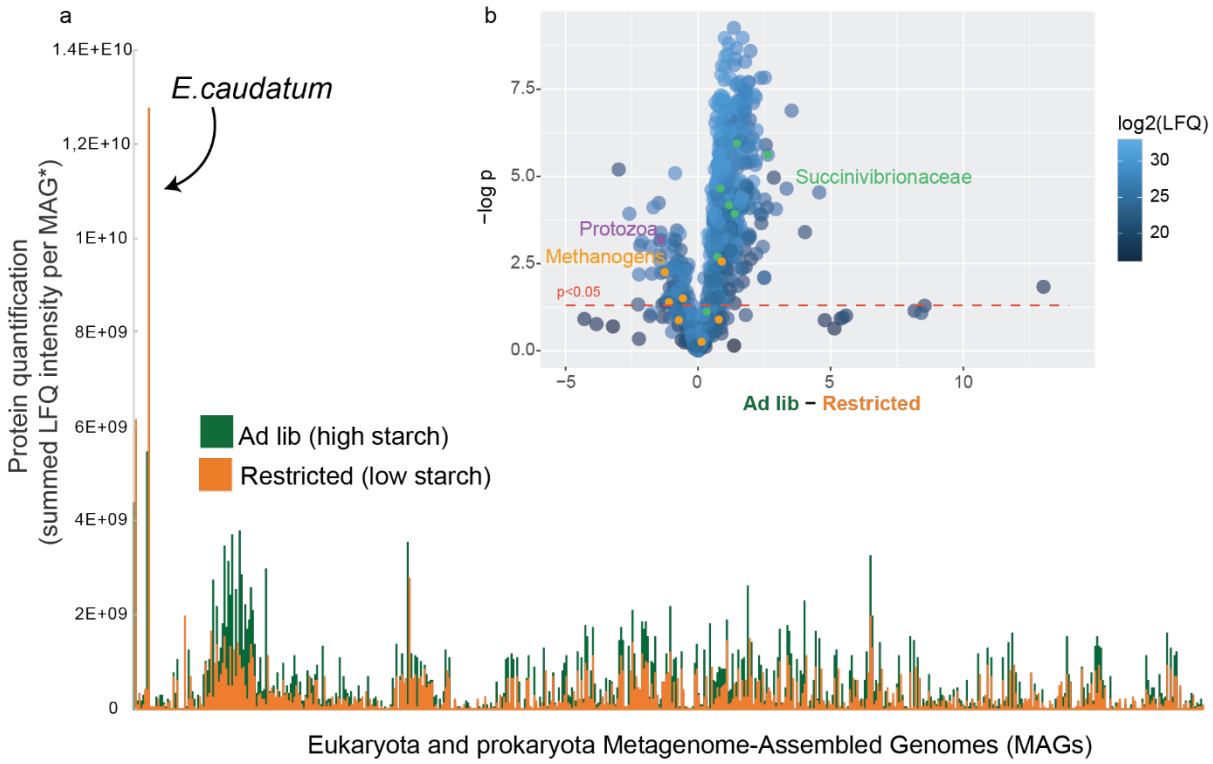

Figure 5. The proteomes of *Entodinium caudatum* and other rumen microbiome populations from Holstein-Friesian beef cattle are affected by high starch diets. A total of 60 beef cattle were subjected to two dietary contrasting condition: 30 animals with *ad libitum* feeding and 30 subjected to 125 days of feed restriction. Dietary components in both treatments consisted of 70% concentrate, and 30% grass silage, with the concentrate containing rolled barley 72.5%, soya 22.5%, molasses 3% and calf mineral 2%. Rolled barley is high in energy and starch content (~50%). **a.** Metaproteomes for a subset of 15 animals (7 restricted and 8 *ad libitium*) were analysed against a database of 781 MAGs and isolate genomes, including eukaryotic representatives such as *E. caudatum*. Protein quantification values (y-axis) were calculated by considering both the number of proteins detected per MAG (and *E. caudatum*) and their LFQ intensity; we averaged LFQ intensities for each detected protein across biological replicates for each dietary condition (Ad lib: green or restricted: orange), which were subsequently summed for all detected proteins per MAG. Similar to our observations in Holstein dairy heifers and Alpine goats (**Figure 3)**, the proteome of *E. caudatum* was a major fraction of the total rumen metaproteome, however it was substantially reduced in dietary conditions where starch content was higher. **b.** Volcano plot indicating different rumen microbiome proteins that displayed both large magnitude of fold-changes in LFQ intensities (x axis) and high statistical significance (-log10 of p values using a t-test, y axis, dashed horizontal line denote a p<0.05 cut-off). Supporting our results in dairy cattle and goats, we observed in high-starch conditions an increase in protein detection for populations affiliated to the *Succinivibrionaceae* (green) compared to the *E. caudatum* (purple) and methanogens (orange), which were detected at higher LFQ intensities in restricted dietary conditions. LFQ intensities used to create panel **a** can be found in **Supplementary Table S4**.

E. caudatum seemingly has preferential bacterial species it will predate.

E. caudatum is renowned for its predatory activity and is acknowledged as the most abundant protozoa in the rumen, whereby it has been estimated that 0.1% of rumen prokaryotes are digested

by the rumen protozoal population every minute [89]. Although suspected of having metabolic interactions with methanogenic archaea, several protozoal populations such as *Entodinium* are hypothesized as having associations with certain members of the Gram negative *Gammaproteobacteria*, which multiple studies have speculated are resistant to protozoal engulfment[74,90,91]. In contrast, Gutierrez and Davis previously demonstrated that *Entodinium*-species engulf Gram positive starch-degraders[91]. In the context of our data, we speculate that CTL fed animals provided *E. caudatum* optimal conditions for predation, whereas increased starch levels in the COS diets facilitated Gram-negative starch-degraders resistant to protozoal engulfment and/or reduced pH levels. Such a scenario would enable populations of *Succinivibrionaceae* in cattle and/or *Aminobacteriaceae* in goats to exploit the "predation free" COS diet and could plausibly explain the observations of higher propionate levels, less methane, and lower activity of *E. caudatum*.

In conclusion, by using a (meta)genome-centric metaproteomics approach we primarily investigated the role of the rumen protozoa *E. caudatum* in the rumen microbiome of beef and dairy cattle as well as dairy goats that were subjected to varying dietary conditions. We showed that the proteome of *E. caudatum* constitutes a substantial fraction of the recovered rumen microbial proteome, which supports previous 16S/18S rRNA gene-based rumen census data that have highlighted its global dominance across a plethora of ruminant species. However, *E. caudatum* proteins were surprisingly detected at lower levels in animals that were fed increased levels of wheat starch, despite its reputable starch-degrading capabilities (**Figures 3-5**). We hypothesize that this scenario is likely caused by the out competition of *E. caudatum* by Gram-negative starch-degrading bacterial species that are possibly resistant to protozoal engulfment and/or lower pH levels, creating sub-optimal conditions for *E. caudatum*. We also observed limited evidence of *E. caudatum* metabolism being directly linked to higher CH_4 yield at the time of sampling in this study (prior to feeding). However, the abundance of *E. caudatum* in high methane-emitting animals may be indirectly fuelled in instances where preferential pH conditions also support methanogens and fibrolytic bacteria that are also known to produce hydrogen. Similarly, our data further support the theories that certain Gram-negative bacterial species are resistant to predation by *E. caudatum*, which could enable specific niches for succinate- and propionate-producing populations to flourish, subsequently exerting a larger impact on hydrogen and methane metabolisms in the rumen microbiome. While much work is still needed to confirm our abovementioned hypotheses, our integrated metaproteomics approaches have demonstrated the future importance of including eukaryote populations for accurate and meaningful analyses of the rumen microbiome and its impact on GHG mitigation strategies and host productivity traits.

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The authors declare no conflicts of interest.

Data Availability

Raw shotgun metagenomic data has been deposited in the National Center for Biotechnology Sequence Read Archive (NCBI-SRA) under accessions numbers SRR19524239 to SRR19524270 with links to BioProject accession number PRJNA844951. All annotated prokaryote MAGs are available publicly at DOI: 10.6084/m9.figshare.20066972.v1. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[92] partner repository with the dataset identifiers PXD034544, PXD034779 and PXD034642.

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