In Vitro Gut Microbiome Response to Carbohydrate Supplementation is Minimally Affected by a Sudden Change in Diet

Ida Gisela Pantoja-Feliciano
U.S. Army (CCDC Soldier Center)
J. Philip Karl
U.S. Army Research Institute of Environmental Medicine (USARIEM)
Matthew Perisin
Combat Capabilities and Development Command – U.S. Army Research Laboratory (CCDC-ARL)
Laurel A. Doherty
U.S. Army (CCDC Soldier Center)
Holly L. McClung
U.S. Army Research Institute of Environmental Medicine (USARIEM)
Nicholes J Armstrong
U.S. Army Research Institute of Environmental Medicine (USARIEM)
Rebecca Renberg
Combat Capabilities and Development Command – U.S. Army Research Laboratory (CCDC-ARL)
Kenneth Racicot
U.S. Army (CCDC Soldier Center)
Tobyn Branck
U.S. Army (CCDC Soldier Center)
Steve Arcidiacono
U.S. Army (CCDC Soldier Center)
Jason W. Soares (jason.w.soares.civ@mail.mil)
U.S. Army (CCDC Soldier Center)

Research Article

Keywords: Gut Microbiome, Carbohydrate metabolism, In vitro fermentation, gut microbiota, microbial ecology, metabolic competition, Microbial Functional Potential, Next Generation Sequencing, Carbohydrate-Active Enzymes, Meal Ready-to-Eat (MRE)

Posted Date: May 26th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-505923/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at BMC Microbiology on January 28th, 2023.
See the published version at https://doi.org/10.1186/s12866-023-02776-2.

Abstract

Background: Interactions between diet, stress and the gut microbiome are of interest as a means to modulate health and performance. Here, *in vitro* fermentation was used to explore the effects of a sudden change in diet, 21 days sole sustenance on the Meal, Ready-to-Eat (MRE) U.S. military combat ration, on inter-species competition and functional potential of the human gut microbiota. Human fecal samples collected before and after MRE intervention or consuming a habitual diet (HAB) were introduced to nutrient-rich media supplemented with starch for *in vitro* fermentation under ascending colon conditions. 16S rRNA amplicon and Whole Genome Sequencing (WGS) were used to measure community composition and functional potential. Specific statistical analyses were implemented to detect changes in relative abundance from taxa, genes and pathways.

Results: Differential changes in relative abundance of five taxa, *Dorea spp., Akkermansia muciniphila*, *Prevotella spp., Desulfovibrio spp.,* and *Dialister spp.,* and four Carbohydrate-Active Enzymes, specifically GH13_14, over the 24 h fermentation were observed as a function of the diet intervention and correlated to specific taxa of interest.

Conclusions: These findings suggest that consuming MRE for 21 days minimally effects changes in gut microbiota structure in response to carbohydrate, but may induce alterations in metabolic capacity. Additionally, these findings demonstrate the potential of starch as a candidate supplemental strategy to functionally modulate specific gut commensals during stress-induced states.

Background:

The human gut microenvironment is influenced by complex interactions between the host and gut microbiome. The network of these interactions is crucial to metabolic processes which maintain gut physiology and host health. Gut microbiota community structure can be modulated by host exposure to different stressors including psychological stress, circadian rhythm disruption, sleep deprivation, environmental factors, and physical activity, which can, in turn, influence host health [1]. Changes in diet are of interest as both a potential source of stress on the gut ecosystem (e.g., a sudden or extreme diet change), but also as an intervention strategy to combat unfavorable stressor-induced shifts in that ecosystem.

Griffin et al. has demonstrated that microbiota responses to a dietary intervention varies among individuals and that metacommunity dynamics (e.g. when an individual's microbiota is connected to other individuals' communities by microbial exchange) can have implications. The group stated that the process of designing probiotic and nutritional interventions includes identifying the microbes associated with specific diets, predicting the responses of the individuals to the diets, and determining if those microbes are related to that dietary practice [2]. It has been shown that modulating the gut microbiota via dietary intervention can reduce symptoms of some metabolic disorders, such as obesity, and associated complications (e.g. systemic inflammation) [3]. Chen et al. reported a literature review focused on gut

microbiota therapeutic interventions using diet as the mechanism to understand the cardioprotective effects on heart failure [4]. Haak et al. reviewed the potential benefits of targeting the microbiota to treat sepsis [5]. Our group recently reported that a sudden diet shift from consuming a habitual diet to consuming the US military Meal, Ready-to-Eat (MRE) combat ration altered fecal microbiota composition, resulting in lower relative abundance of multiple genera of lactic acid bacteria (e.g. *Lactobacillus, Lactococcus, Leuconostoc*) and increased relative abundance of several saccharolytic genera (*Streptococcus* and *Clostridium*) [6]. An *in vitro* fermentation experiment was also used to assess the potential use of carbohydrate, namely resistant starch (RS2) for restoring *Lactobacillus* following MRE consumption. That approach allowed inter-species competition in samples collected before and after the MRE intervention and at the same time points in a control group to be studied over the time scale of hours, which is not feasible in *in vivo* human studies which commonly rely on daily stool samples. *Ruminococcus bromii*, a keystone taxa and resistant starch degrader, increased in relative abundance during the MRE diet in the presence of RS2 while the ability of *Lactobacillus* to compete in presence of RS2 appeared to be reduced [7]. However, those results were limited in that only a few selected taxa were measured, the identity of which *Lactobacillus* species affected could not be elucidated, and differences in functional capacity of the community could not be examined nor the whole community metabolic response to RS both compositionally and functionally. The results did provide initial insight into how *in vitro* studies can complement human study results and reveal microbial community functional understanding in response to stress.

Herein we report a comprehensive genomic analysis employing both 16S rRNA gene amplicon and whole genome sequencing (WGS) of samples collected during the *in vitro* fermentation experiment described in Pantoja-Feliciano et al 2019 [7] to reveal influence of a sudden change in diet on whole bacterial community composition and functional potential. To explore stress-induced microbial community responses, carbohydrate content, specifically RS, in medium was increased five-fold to allow the study of nutrient:microbiome interactions that cannot easily be explored *in vivo* [7].

Results:

Stressor-induced changes to microbial composition:

Random Forest analysis was used to identify a subset of the community driving differences in community composition (**Figure 3**). For this analysis, features that were present in less than half of the samples in all diet-day combinations at 0 h (start of fermentation) were removed and the fermentation time points data (0, 5, 10 and 24 h) were combined, resulting in an analysis as a function of the diet and study day.15% of the genera (30 out 201 total) were identified for all the groups, but a differentiation by taxa and diet groups was not obtained. For that, a linear mixed model analysis using a multivariate ANOVA with repeated measures on the identified 30 taxa was employed to detect abundance variations as a function of the MRE-diet intervention (Diet*StudyDay*Fermentation Time interaction).

Out of the remaining taxa derived from Random Forest analysis, five taxa, *Dorea spp., Akkermansia muciniphila*, *Prevotella spp., Desulfovibrio spp.,* and *Dialister spp.,* were identified as having a statistically significant interaction between diet, study day, and fermentation residence time (p-values 0.0061, 0.0147, 0.0004, 0.0198, 0.0002 respectively), (**Figure 4**). *Dorea spp*. notably increases in the MRE day 21 group after 10 h of exposure to starch-supplemented medium relative to the other groups (**Figure 4A)**. *Akkermansia muciniphila* was diminished in relative abundance in MRE day 21 compared to HAB diet day 0 and 21 and MRE day 0 throughout the fermentation; however, the rate of change over the course of fermentation differed in MRE day 21 compared to MRE day 0 ($p<0.001$) as determined by an equality of slopes test (**Figure 4B, Figure S4B**). A similar case was observed for *Prevotella spp*. where abundance at inoculation was higher but decreased as the fermentation proceeded (**Figure 4C)**; however, differences between MRE day 0 and MRE day 21 were not detected at later time-points, and after 24 h residence time, this organism was completely diminished in both groups. *Desulfovibrio spp*. abundance was higher at the beginning of the fermentation in the MRE day 21 group relative to the other groups but decreased more rapidly during fermentation ($p>0.001$) such that no difference was observed between groups after 24 h (**Figure 4D, Figure S4D**). *Dialister* in MRE 21 was significantly different than that of HAB 0, HAB 21, or MRE 0 at multiple time-points, and an equality of slopes test further indicated differential growth over the course of fermentation ($p<0.001$) (**Figure S4E**). Because changes between HAB 0 and HAB 21 were also observed, we cannot conclusively state that *Dialister's* growth in MRE 21 is due to the all-MRE diet. (**Figure 4E**). See supplementary **Tables S1** and **S2** for more details about the p-values and completed analysis.

Stressor-induced changes to Microbial Functional Potential

Whole genome sequencing was employed to complement 16S analyses and explore the influence of MRE-diet intervention at a functional level. We obtained a total number of raw reads of 245 357 634, including both paired reads; a total number of QC reads of 245 000 244; a mean raw reads per sample of 3 774 732; and a mean QC reads per sample of 3 769 234.

Though the diet*study day interaction only resulted in subtle alterations in community composition as assessed by 16S sequencing, genomes for strains that have nearly identical ribosomal RNA sequences have been shown to possess different functional capabilities [8]. Using the assembly free program HUMAnN2 [9], we assessed community wide function and observed similar clustering patterns as those seen with 16S. PCoA of Bray-Curtis distances in gene family abundances (**Figure S5A**) resulted in the first principal coordinate associating with fermentation time (PERMANOVA, $R^2 = 0.23$, $p = 0.001$). The second principal coordinate was associated with diet (PERMANOVA, $R^2 = 0.17$, $p = 0.001$) and there was a small effect of Diet*Date (PERMANOVA, $R^2 = 0.03$, $p = 0.001$). A pathway functional level (**Figure S5B**), PCoA of Bray-Curtis distances in pathway abundances primarily clustered by fermentation time (PERMANOVA, $R^2 = 0.31$, $p = 0.001$) with effects of Diet (PERMANOVA, $R^2 = 0.15$, $p = 0.001$) and Diet*Date (PERMANOVA, $R^2 = 0.02$, $p = 0.001$).

To parse whether there were finer scale differences for specific strains or functions, sequences from all samples were co-assembled, binned into metagenome assembled genomes (MAGs), and functionally

annotated. After binning, we assembled 120 MAGs at >50% completion and <10% contamination including 57 MAGs at >90% completion and <5% contamination and 63 MAGs at >50% completion, <10% contamination. PCoA of Bray-Curtis distances for MAG abundances showed similar clustering by diet and fermentation residence time to the 16S PCoA (**Figure S6A)**. The first principal coordinate was associated with fermentation time (PERMANOVA, R^2 = 0.27, p = 0.001) and the second with diet (PERMANOVA, R^2 = 0.23, p = 0.001). There was a small, significant effect of Diet*Date (PERMANOVA, R^2 = 0.03, p = 0.001). Thus, at the MAG level, there was not a large effect of MRE diet when comparing Day 0 to Day 21.

Due to the inclusion of starch supplementation during fermentation, we assessed whether specific functions for complex carbohydrate breakdown (Carbohydrate-active enzymes, CAZymes) were affected by Diet*Date. Hidden Markov models were used to identify and classify CAZymes in the metagenome assembly. PCoA of Bray-Curtis distances for CAZyme abundances showed similar clustering patterns to the pathway PCoA (**Figure S6B**). The first principal component was primarily associated with fermentation time (PERMANOVA, R^2 = 0.57, p = 0.001) with small effects of Diet (PERMANOVA, R^2 = 0.06, p = 0.001) and Diet*Date (PERMANOVA, R^2 = 0.03, p = 0.001). To uncover CAZymes which were important for classifying the Diet*Date categories, we employed Random Forests and linear mixed models (**Figure 5)**. Four CAZymes passed the significance threshold: GH13_14 (pullulanase), GT76 (Dol-P-Man: α-1, 6-mannosyltransferase), CBM83 (starch binding), and CBM27 (mannan binding) (**Table S3** and **Figure 5B**). **Table S4** shows the p-values corresponding to the pairwise multiple comparison analysis Tukey HSD for each fermentation time point in the different groups, supporting **Figure 5B**. In the case of GH13_14, MRE day 21 group is significantly different from the other groups at 10 and 24 h after fermentation. GH13_14 was of particular interest because these enzymes catalyze the cleavage of branched RS2 breakdown products. MAG and taxonomic breakdown of GH13_14 by Diet*Date indicated that the increased abundance in MRE Day 21 samples was due to a *Coproccocus comes* MAG (**Figure 6A**). Another interesting case was the CAZyme GT76 and its prevalence in the MRE day 21 group associated with *Lachnospira eligens* (**Figure 6B**). Thus, the MRE diet did result in subtle functional differences at the fine-scale CAZyme level. Similar analysis was employed to find pathways that were important for differentiating Diet*Date categories, but only minor effects of Diet*Date were evident (**Figure S7A**, **Table S5** and **Figure S7B**).

Discussion

This study used an *in vitro* fermentation system that simulated the conditions of the gut, to examine the effects of starch supplementation on gut microbial community composition and functional capacity in samples collected from volunteers that consumed two different diets, the Meal, Ready-to-Eat (MRE) U.S. military combat ration and a habitual diet (HAB) for 21 days. Only subtle changes in gut microbiota structure and metabolic capacity in response to RS2 were observed as a result of MRE diet consumption suggesting that the MRE diet does not substantially influence competitive dynamics within the gut microbiome for the model substrate.

Several studies have identified changes in microbial communities due to RS consumption. Martinez et al has previously shown fecal microbiota composition changes after a RS2 diet in a human study, specifically a significant increase in the *Ruminococcus bromii* and *Eubacterium rectale* proportions [10]. A more recent study reported gut microbiota changes in mice when introducing a RS diet. Researchers observed a significant increase in members of the *Proteobacteria* and *Verrucromicrobia* phyla correlated to an observed increase in anxiety-like behaviors within the animals [11]. These are contrary to our results in which *Desulfovibrio* and *Akkermansia muciniphila* decreased upon exposure to RS-supplemented medium. This discrepancy may be due to differences in study design, comparing results from human samples to an animal model results, fermentation conditions and interindividual variability of the volunteers.

Although ~ 200 taxa were identified by 16S rRNA sequencing, only 5 bacterial groups (out of 30 after Random Forest) showed differential changes in relative abundance during the fermentations as a function of the diet intervention. These groups, *Prevotella spp.*, *Desulfovibrio spp.*, *Dorea spp.*, *Dialister spp*, and *Akkermansia muciniphila*, are different than those identified in the previously reported qPCR analysis from the equivalent sample set [7]. Reasons for the inconsistency are unknown, but may include the use of 16S and metagenomic analysis in this study rather than using qPCR to targeted specific species as was done in the previous study. The five taxa identified by 16S in the present analysis, however, represent gut commensals that have a range of clinical and physiological relevance. For two of them, *Dorea spp.* and *Dialister spp.*, inter-species competition for RS were significantly altered following 21d on the MRE diet. *Dorea spp* are members of the Clostridium cluster XIVa and are a dominant species in the human gut [12]. *Dorea spp* utilizes dietary carbohydrates such as simple sugars (eg, glucose, lactose, maltose), inulin and fructo-oligosaccharides to produce metabolic products including acetate, formate, lactate and ethanol [13]. Although some species are unable to directly metabolize starch, it is associated with starch absorption in mice, perhaps through cross-feeding on small hydrolysis products (e.g., maltose, glucose) from initial starch degradation by starch-degrading taxa [14], including possibly Prevotella whose abundance was increased due to the MRE-diet relative to the HAB diet. *Dorea* is considered part of a healthy gut microbiota, although it has also been shown to have increased abundance in Multiple Sclerosis and IBD patients [15]. *Dialister* is composed of a small number of species, with only *Dialister succinatiphilus* has been isolated from feces [16]. *Dialister* relative abundance is reportedly increased by consuming walnuts [17], almonds [18] and whole grains [19]. These effects may be beneficial to health as *Dialister* produces propionate [20] and increases in relative abundance has been associated with reduction of IL-6 levels in response to whole grains [19] and lower incidence of depression [21]. However, increased abundance has also been associated with obesity and inability to lose weight [22], gastrointestinal disease (IBS, UC, CD) [23] and Alzheimer's [24]. Taken together, it is not clear whether the effect of the MRE diet on *Dorea* and *Dialister* responses to RS2 we observed in this study can be considered beneficial for the microbial community or host. For three other taxa, *Prevotella spp.*, *Desulfovibrio spp.*, and *Akkermansia muciniphila*, relative abundance differed in the MRE-day 21 samples at the 0h time point, but changes over time during the fermentation did not differ as a function of diet. This suggests an effect of the MRE diet on those taxa, but not the response of those taxa to RS2.

Though that result does not match the previous 16S compositional analysis from the human study [6], this is likely due to only a sub-population (n = 5 volunteers per group) being used in the *in vitro* experiment rather than all 30 study volunteers. The subtle distinctions within the findings are difficult to draw conclusions associated with these taxa. *Prevotella* spp. is thought to be beneficial due to its prevalence in a high fiber diets and has also been shown at the family level to increase following high RS diet [25] and during *in vitro* fermentation studies [26], [27]. *A. muciniphila* has been previously associated with beneficial health outcomes [28];[29] and has been shown to metabolically respond to high RS diet in rats [30] and humans [25], while *Desulfovibrio* spp. has been associated with intestinal inflammation [31]. These taxa warrant further investigation as bacterial targets for RS supplementation.

WGS analysis identified two CAZymes, GH13_14 and GT76, that differentially changed in response to RS following MRE consumption relative to other samples. Enrichment of the extracellular glycan-active enzyme glycoside hydrolase (GH13_14) was due to *Coprococcus comes*, a member of the Clostridium cluster XIVa [32]. GH13_14 is a pullulanase common in human gut lactobacilli. As a butyrate producer, *Coprococcus comes* is generally thought to be beneficial. It has also been negatively correlated in type 1 diabetes patients [33]. Maier et al has shown CAZymes and transport systems related to *C. comes* have increased in abundance in response to an RS diet [25]. *Lachnospira eligens* was related to the CAZyme GT76. *L. eligens* utilizes pectin and polygalacturonic acid, with acetate, formate, ethanol, and CO2 as major end products [34]. It has been associated with the glycosyltransferase GT76, a α-1, 6-mannosyltransferase that uses dolichol-P-mannose as a sugar donor. GT's are enzymes that forms glycosidic bonds and are involved with biosynthesis of di-, oligo-, and polysaccharides (www.cazy.com). Both *C. comes and L eligens* were not directly identified in the 16S rRNA analysis at the species level but the genus *Coprococcus* and *Lachnospira* were part of the 30 most abundant taxa. These groups were not significant in the random forest analysis, which may suggest that not all species within each genus respond in the same way and also highlights the value of a higher level of resolution provided by WGS compositional and functional analysis. Otherwise, these taxa significantly contributed to CAZyme alterations and community carbohydrate metabolism in the presence of RS2. For CAZymes CBM83 and CBM27, the 3-way interactions analysis are being driven by an unexpected response in HAB d21. Therefore, it is not clear that these responses are truly a result of MRE consumption.

The study was limited by use of only a subset of volunteers within the *in vitro* fermentation studies pooling the samples limited individualized study outcomes and a lack of correlative SCFA, metabolomics and proteomics analysis to corroborate bioinformatics findings. However, the data does demonstrate sudden changes in diet had a functional effect on community competition for RS and that certain potentially beneficial taxa respond to RS supplementation differentially as a function of diet and stress.

Conclusion

In this study, we used *in vitro* fermentation to explore the effects of an acute stressor, a sudden change in diet from habitual to sole sustenance on MREs, on inter-species competition dynamics of gut microbiome in response to starch supplementation. The main finding of this study was that MRE consumption does

not appear to substantially impact the effects of RS2 on the gut microbiome. Rather, only minimal alterations in community composition and functional potential as measured by CAZyme relative abundance were observed. These results did demonstrate community metabolic capacity and competition for substrates can be altered even when taxa abundance are not significantly different in the absence of that substrate. The findings demonstrate the value of combining human microbiome studies, *in vitro* fermentation, and powerful next generation sequencing techniques like 16S and WGS, to effectively gain a more complete understanding of the effects of stress on competitive nutrient:microbiome:interactions and to identify potential strategies toward modulating gut commensal metabolic competition during stress states.

Methods

Participants

Fecal samples were collected from ten individuals participating in a randomized controlled trial designed to determine the effects of subsisting on a MRE-only diet on gut microbiota composition and intestinal permeability [6]. For more information about the characteristics of the participants and their diets see Supplemental Table 1 from Pantoja-Feliciano et al 2019. Study details and primary findings have been previously reported (Karl 2019). Briefly, the full study population included 64 adults without obesity, 18–62 year who were randomly assigned to follow their normal habitual diet for 21d (HAB) or consume a provided diet containing only the MRE rations for 21d. Study exclusion criteria included: use of antibiotics or colonoscopy within 3 mo of enrollment, vegetarian diet, history of gastrointestinal (GI) disease, infrequent bowel movements (< 4x/wk), and habitual use of medications affecting GI function (e.g. laxatives, anti-diarrheals). All participants were instructed to discontinue use of probiotic, prebiotic, or other dietary supplements ≥ 2 wk before study participation. Study involvement was voluntary, and written informed consent was obtained prior to enrollment. The study was reviewed and approved by the US Army Research Institute of Environmental Medicine Human Institutional Review Board (Natick, MA). Investigators adhered to the policies regarding the protection of human subjects as prescribed in Army Regulation 70 – 25, and the research was conducted in adherence with the provisions of 32 CFR Part 219. The trial is registered on www.clinicaltrials.gov as NCT02423551.

Fecal Samples

Fecal samples were collected at baseline (day 0) and at the end of the 21d MRE intervention period (day 21). Samples were collected into provided 650 mL collection containers to which an anaerobic sachet (GasPak EZ Anaerobe Container System; Becton, Dickinson and Co., Franklin Lakes, NJ) was immediately added. The sealed container was then kept on ice or in a refrigerator until processing [35]. Fecal slurry (20%) was prepared within 12 h of donation by addition of 0.1M phosphate buffer pH 7.2 supplemented with 15% w/v glycerol and 0.08% L-cysteine (Sigma-Aldrich; St. Louis, MO), to fresh feces in a 4:1 ratio, followed by homogenizing for two minutes in a Seward Ltd. Model 400 stomacher (Davie, FL). The slurry was anaerobically divided into aliquots and stored at -80°C until needed.

Fermentation System Protocol (Scheme 1)

All chemicals were obtained from Sigma-Aldrich unless otherwise indicated. The fermentation parameters are outlined in Pantoja-Feliciano et al [7]. Briefly, fermentation medium was prepared based on Macfarlane et al.[36] with the following modifications: addition of resazurin (1 ug/L) and supplemented with a 5-fold increase in potato resistant starch (15g/L, RS). After mixing well, the nutrient-rich medium was added to fermentation vessels (125 mL/vessel) autoclaved, equilibrated overnight under constant headspace flush with oxygen-free N_2 (20psig, 5 mL/min) and adjusted to emulate the ascending colon (pH 5.5). Fecal samples collected from ten individuals participating in the parent study on day 0 and day 21 (HAB, n = 5; MRE, n = 5) were pooled in equal proportions and vessels inoculated with 10% ($^{v}/_{v}$) fecal slurry for final 2% inocula ($^{w}/_{v}$). Pooling promotes a highly diverse community and allows incorporation of low abundant, keystone species that may be limited using individualized samples to generate more generalizable insight. Parallel control vessels were inoculated with cell-free phosphate buffer/glycerol. Aliquots were temporally removed from each vessel at 0, 5, 10, 24 and 48h after exposure to RS2-supplemented medium and stored at -80^{o}C for DNA extraction and sequencing analysis. Fermentations were run in triplicate, at the same time, as experimental replicates.

16S rRNA gene amplicon sequencing

DNA from fecal samples was extracted using the QIAMP Power Fecal DNA Extraction Kit, QIAGEN, Inc. (Germantown, MD). DNA concentration (ng/uL) was quantified using Nanodrop One^{c} (ThermoFisher Scientific, Inc., Waltham, MA). Primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT) were used to amplify the V4 region of the 16S rRNA gene [37, 38]. No barcodes or adapters were included in the primers. Instead, the two-step, dual index PCR approach was used [39]. Nextera XT Index Kit v2 sets A and B (Cat. Nos. FC-131-2001 and FC-131-2002, Illumina, CA, USA) were used to index the 16S amplicons. The libraries were then normalized, pooled, and paired end sequenced (2x150bp) on a NextSeq 500 (Illumina, CA, USA).

For downstream analyses, only the first forward sequencing reads were used. Primers were removed with the Python package cutadapt and only sequences 100 nucleotides or more were retained [40]. QIIME2 with the DADA2 plugin (*qiime dada2 denoise-single*) was used to assign amplicon sequence variants (ASVs) with the added parameter of truncating sequences at position 124 [41, 42]. A phylogenetic tree was constructed with FastTree [43] by using the *qiime alignment maft*, *qiime alignment mask*, *qiime phylogeny fasttree*, and *qiime phylogeny midpoint-root* plugins, all with default parameters. Taxonomy was assigned using Greengenes (gg-13-8-99-515-806-nb-classifier).

Whole-genome sequencing

Sequencing libraries were prepared using the Nextera XT DNA Library Prep Kit (Cat. No. FC-131-1096, Illumina, CA, USA) and the Nextera XT Index Kit v2 set C (Cat. No. FC-131-2003, Illumina, CA, USA)

according to the manufacturer's protocol. Samples were normalized, pooled, and paired-end sequenced (2x150bp) on a NextSeq500 (Illumina, CA, USA).

Metagenomic assembly and binning was completed with metaWRAP pipeline modules [44]. Default parameters were used unless noted. The module *metawrap read-qc* was used to quality filter reads for each sample. Paired-end reads for all samples were combined and co-assembled with the *metawrap assembly* module using MEGAHIT [45, 46]. Assembled contigs were binned with the *metawrap binning* module using MetaBAT2, MaxBin2, and CONCOCT programs [47–49]. Bins were consolidated and refined with the *metawrap bin_refinement* module with a minimum completion of 50% and maximum contamination of 10%. Bin abundances across samples were quantified with the *metawrap quant_bins* module which uses Salmon [50]. To improve assemblies, the refined bins were reassembled with the *metawrap reassemble_bins* module with a minimum completion of 50% and maximum contamination of 10%. Reassembled bins were functionally annotated with the *metawrap annotate* module which uses Prokka [51]. To search the metagenome assembly for CAZymes, the *run_dbcan.py* (https://github.com/linnabrown/run_dbcan) script was used. This script uses hidden Markov models (HMM) to search for CAZyme boundaries according to the dbCAN CAZyme domain HMM database [52, 53]. Finally, bin taxonomy was assigned according to The Genome Taxonomy Database (GTDB) [54]. First, the reassembled bins were converted into contig databases with Anvi'o (*anvi-script-reformat fasta*, *anvi-gen-contigs-database* programs) [55]. Single-copy core gene taxonomy search databases were setup with the *anvi-scg-databases* program and taxonomy was estimated using the *anvi-run-scg-taxonomy* and *anvi-estimate-genome-taxonomy* programs. For functional and taxonomic analyses independent of metagenome assembly, HUMAnN2 [9] and MetaPhlan2 [56] were used to annotate gene families/pathways and taxonomy, respectively.

The combination of both techniques (16S and WGS) can contribute to comprehending the differences within and between individuals/samples [57]. By employing 16S analysis, community composition changes were explored while with whole genome sequencing, functional capacity of the community in terms of genes and pathways were examined. The advantage of using 16S for the taxonomic identification is the large and comprehensive availability of reference databases leading to more accurate results [58]. Both techniques can be used as a complement of each other as they provide powerful combined information.

Data analysis

Custom R [59] scripts were used for analysis and visualization of 16S and whole genome data. For principal coordinates analysis (PCoA), the *vegan* package (https://github.com/vegandevs/vegan/) was used to compute Bray-Curtis distances between samples and this distance matrix was input into PCoA with the *labdsv* package (http://ecology.msu.montana.edu/labdsv/R). Experimental factor significance and proportion of variance explained was determined by PERMANOVA with the *adonis* function using the Bray-Curtis distance matrix. For Random Forest analysis, features that were present in less than half of the samples were removed. The data was baselined by subtracting feature abundance for each bioreactor vessel at time zero from feature abundances at subsequent time points. To determine

importance and significance for Diet*Date features, the *randomForest* function (https://www.stat.berkeley.edu/~breiman/RandomForests/) was used with ntree = 10001 using data for fermentation time points 0, 5, 10 and 24 hours. *RColorBrewer* (https://cran.rproject.org/web/packages/RColorBrewer/index.html) and *ggplot2* [60] were used for visualizations.

Raw sequencing reads and metagenome assembled data were deposited in the public database NCBI SRA, BioProject ID: PRJNA675102 (https://www.ncbi.nlm.nih.gov/search/all/?term=PRJNA675102).

Statistical Analysis

Relative abundance data for the top 30 organisms, 30 pathways, and 30 CAZymes found to be important for differentiating diet*date by Random Forest analysis in R software were arcsine square root transformed to normalize distributions and analyzed using repeated measured ANOVA. Models included with “Diet” (MRE and HAB) and “Study day” (0 and 21) as between-groups factors and “Fermentation time” (0, 5, 10, 24 and 48h) as a within-subjects factor. Validity of repeated measures results for each organism was assessed by testing for sphericity. For organism data that passed the test for sphericity, p-values of main effects as well as two- and three-way interactions were generated using an unadjusted univariate F-test; in cases where the test for sphericity failed, a Greenhouse-Geisser (G-G) epsilon adjusted F-test was used to generate p-values.

For all features demonstrating a statistically significant diet*study day*fermentation time interaction ($p < 0.05$), pairwise comparisons between groups were tested within each time point separately using ANOVA with Tukeys HSD. To assess whether significant differences were due to relative abundance at 0h, features were subjected to analysis of covariance with fermentation time as the covariate and tested for equality of slopes between groups ($p < 0.05$) (one-way ANCOVA). R and SigmaStat software was used for analysis.

Abbreviations

MRE: Meal Ready-to-Eat; HAB:Habitual Diet; RS2:Resistant Starch II; RS:Resistant Starch; PD:Faith’s Phylogenetic Diversity; MAGs:Metagenome Assembled Genomes; CAZymes:Carbohydrate-active Enzymes; WGS:Whole Genome Sequencing

Declarations

Ethics approval and consent to participate: The study was reviewed and approved by the US Army Research Institute of Environmental Medicine Human Institutional Review Board (Natick, MA). Investigators adhered to the policies regarding the protection of human subjects as prescribed in Army Regulation 70-25, and the research was conducted in adherence with the provisions of 32 CFR Part 219. The trial is registered on www.clinicaltrials.gov as NCT02423551.

Consent for publication: Not applicable

Availability of data and materials: Raw sequencing reads and metagenome assembled data were deposited in the public database NCBI SRA, BioProject ID: PRJNA675102.

Competing interests: The authors declare that they have no competing interests.

F**unding**: This work was supported by the US Defense Health Program Joint Program Committee 5 (Program JPC-5). The funding and overall program oversight was through this Program.

Authors' contributions: IGPF: Analyzed the data, contributed with analysis tools and in writing the paper; JPK: contributed in the design of experiments, helped in the analysis of the data, contributed with samples and in writing the paper; MP: contributed in the analysis of the data and with analysis tools; LAD: contributed performing the experiments, in the analysis of the data, and with analysis tools; HLM: contributed performing the experiments (helped in the human portion); NJA: contributed performing the experiments (helped in the human portion); RR: contributed with reagents/materials/analysis tools; KR: contributed in the design of the experiments; TB: helped conduct the *in vitro* experiments; SA: contributed in writing the paper; JWS: conceived and designed the experiments, performed the experiments, helped in the analysis of the data, contributed with reagents/materials/analysis tools, writing the paper and is the corresponding author. All authors have read and approved the manuscript.

Acknowledgments: We acknowledge the technical support of SGT Alfonzo Patino, Claire Whitney, Patrick Radcliffe, and Anthony Karis, and the support provided by the IT Specialists at DREN - CCDC - Soldier Center, IMO - G6.

Disclaimer: The views expressed are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the U.S. Government. Any citations of commercial organizations and trade names do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations. Approved for public release; distribution is unlimited.

References

1. Karl JP, Hatch AM, Arcidiacono SM, Pearce SC, Pantoja-Feliciano IG, Doherty LA, Soares JW: **Effects of Psychological, Environmental and Physical Stressors on the Gut Microbiota**. *Front Microbiol* 2018, **9**:2013.
2. Griffin NW, Ahern PP, Cheng J, Heath AC, Ilkayeva O, Newgard CB, Fontana L, Gordon JI: **Prior Dietary Practices and Connections to a Human Gut Microbial Metacommunity Alter Responses to Diet Interventions**. *Cell Host Microbe* 2017, **21**(1):84-96.
3. Xiao S, Fei N, Pang X, Shen J, Wang L, Zhang B, Zhang M, Zhang X, Zhang C, Li M *et al*: **A gut microbiota-targeted dietary intervention for amelioration of chronic inflammation underlying metabolic syndrome**. *FEMS Microbiol Ecol* 2014, **87**(2):357-367.

4. Chen X, Li HY, Hu XM, Zhang Y, Zhang SY: **Current understanding of gut microbiota alterations and related therapeutic intervention strategies in heart failure**. *Chin Med J (Engl)* 2019, **132**(15):1843-1855.
5. Haak BW, Prescott HC, Wiersinga WJ: **Therapeutic Potential of the Gut Microbiota in the Prevention and Treatment of Sepsis**. *Front Immunol* 2018, **9**:2042.
6. Karl JP, Armstrong NJ, McClung HL, Player RA, Rood JC, Racicot K, Soares JW, Montain SJ: **A diet of U.S. military food rations alters gut microbiota composition and does not increase intestinal permeability**. *J Nutr Biochem* 2019, **72**:108217.
7. Pantoja-Feliciano IG, Soares JW, Doherty LA, Karl JP, McClung HL, Armstrong NJ, Branck TA, Arcidiacono S: **Acute stressor alters inter-species microbial competition for resistant starch-supplemented medium**. *Gut Microbes* 2019, **10**(4):439-446.
8. Eren AM, Morrison HG, Lescault PJ, Reveillaud J, Vineis JH, Sogin ML: **Minimum entropy decomposition: unsupervised oligotyping for sensitive partitioning of high-throughput marker gene sequences**. *ISME J* 2015, **9**(4):968-979.
9. Franzosa EA, McIver LJ, Rahnavard G, Thompson LR, Schirmer M, Weingart G, Lipson KS, Knight R, Caporaso JG, Segata N *et al*: **Species-level functional profiling of metagenomes and metatranscriptomes**. *Nat Methods* 2018, **15**(11):962-968.
10. Martinez I, Kim J, Duffy PR, Schlegel VL, Walter J: **Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects**. *PLoS One* 2010, **5**(11):e15046.
11. Lyte M, Chapel A, Lyte JM, Ai Y, Proctor A, Jane JL, Phillips GJ: **Resistant Starch Alters the Microbiota-Gut Brain Axis: Implications for Dietary Modulation of Behavior**. *PLoS One* 2016, **11**(1):e0146406.
12. Cecchini DA, Laville E, Laguerre S, Robe P, Leclerc M, Dore J, Henrissat B, Remaud-Simeon M, Monsan P, Potocki-Veronese G: **Functional metagenomics reveals novel pathways of prebiotic breakdown by human gut bacteria**. *PLoS One* 2013, **8**(9):e72766.
13. Taras D, Simmering R, Collins MD, Lawson PA, Blaut M: **Reclassification of Eubacterium formicigenerans Holdeman and Moore 1974 as Dorea formicigenerans gen. nov., comb. nov., and description of Dorea longicatena sp. nov., isolated from human faeces**. *Int J Syst Evol Microbiol* 2002, **52**(Pt 2):423-428.
14. Herrmann E, Young W, Reichert-Grimm V, Weis S, Riedel CU, Rosendale D, Stoklosinski H, Hunt M, Egert M: **In Vivo Assessment of Resistant Starch Degradation by the Caecal Microbiota of Mice Using RNA-Based Stable Isotope Probing-A Proof-of-Principle Study**. *Nutrients* 2018, **10**(2).
15. Chen J, Chia N, Kalari KR, Yao JZ, Novotna M, Paz Soldan MM, Luckey DH, Marietta EV, Jeraldo PR, Chen X *et al*: **Multiple sclerosis patients have a distinct gut microbiota compared to healthy controls**. *Sci Rep* 2016, **6**:28484.
16. Morotomi M, Nagai F, Sakon H, Tanaka R: **Dialister succinatiphilus sp. nov. and Barnesiella intestinihominis sp. nov., isolated from human faeces**. *Int J Syst Evol Microbiol* 2008, **58**(Pt 12):2716-2720.

17. Holscher HD, Guetterman HM, Swanson KS, An R, Matthan NR, Lichtenstein AH, Novotny JA, Baer DJ: **Walnut Consumption Alters the Gastrointestinal Microbiota, Microbially Derived Secondary Bile Acids, and Health Markers in Healthy Adults: A Randomized Controlled Trial**. *J Nutr* 2018, **148**(6):861-867.
18. Holscher HD, Taylor AM, Swanson KS, Novotny JA, Baer DJ: **Almond Consumption and Processing Affects the Composition of the Gastrointestinal Microbiota of Healthy Adult Men and Women: A Randomized Controlled Trial**. *Nutrients* 2018, **10**(2).
19. Martinez I, Lattimer JM, Hubach KL, Case JA, Yang J, Weber CG, Louk JA, Rose DJ, Kyureghian G, Peterson DA *et al*: **Gut microbiome composition is linked to whole grain-induced immunological improvements**. *ISME J* 2013, **7**(2):269-280.
20. Koh A, De Vadder F, Kovatcheva-Datchary P, Backhed F: **From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites**. *Cell* 2016, **165**(6):1332-1345.
21. Valles-Colomer M, Falony G, Darzi Y, Tigchelaar EF, Wang J, Tito RY, Schiweck C, Kurilshikov A, Joossens M, Wijmenga C *et al*: **The neuroactive potential of the human gut microbiota in quality of life and depression**. *Nat Microbiol* 2019, **4**(4):623-632.
22. Muniz Pedrogo DA, Jensen MD, Van Dyke CT, Murray JA, Woods JA, Chen J, Kashyap PC, Nehra V: **Gut Microbial Carbohydrate Metabolism Hinders Weight Loss in Overweight Adults Undergoing Lifestyle Intervention With a Volumetric Diet**. *Mayo Clin Proc* 2018, **93**(8):1104-1110.
23. Xiang-qian Wang A-hZ, Jian-hua Miao, Hui Sun, Guang-li Yan, Fang-fang Wu and Xi-jun Wang: **Gut microbiota as important modulator of metabolism in health and disease**. *The Royal Society of Chemistry* 2018, **8**:42380–42389.
24. Vogt NM, Kerby RL, Dill-McFarland KA, Harding SJ, Merluzzi AP, Johnson SC, Carlsson CM, Asthana S, Zetterberg H, Blennow K *et al*: **Gut microbiome alterations in Alzheimer's disease**. *Sci Rep* 2017, **7**(1):13537.
25. Maier TV, Lucio M, Lee LH, VerBerkmoes NC, Brislawn CJ, Bernhardt J, Lamendella R, McDermott JE, Bergeron N, Heinzmann SS *et al*: **Impact of Dietary Resistant Starch on the Human Gut Microbiome, Metaproteome, and Metabolome**. *mBio* 2017, **8**(5).
26. Wang Y, Mortimer EK, Katundu KGH, Kalanga N, Leong LEX, Gopalsamy GL, Christophersen CT, Richard AC, Shivasami A, Abell GCJ *et al*: **The Capacity of the Fecal Microbiota From Malawian Infants to Ferment Resistant Starch**. *Front Microbiol* 2019, **10**:1459.
27. Warren FJ, Fukuma NM, Mikkelsen D, Flanagan BM, Williams BA, Lisle AT, P OC, Morrison M, Gidley MJ: **Food Starch Structure Impacts Gut Microbiome Composition**. *mSphere* 2018, **3**(3).
28. Png CW, Linden SK, Gilshenan KS, Zoetendal EG, McSweeney CS, Sly LI, McGuckin MA, Florin TH: **Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria**. *Am J Gastroenterol* 2010, **105**(11):2420-2428.
29. Rajilic-Stojanovic M, Shanahan F, Guarner F, de Vos WM: **Phylogenetic analysis of dysbiosis in ulcerative colitis during remission**. *Inflamm Bowel Dis* 2013, **19**(3):481-488.

30. Keenan MJ, Janes M, Robert J, Martin RJ, Raggio AM, McCutcheon KL, Pelkman C, Tulley R, Goita M, Durham HA *et al*: **Resistant starch from high amylose maize (HAM-RS2) reduces body fat and increases gut bacteria in ovariectomized (OVX) rats**. *Obesity (Silver Spring)* 2013, **21**(5):981-984.
31. Loubinoux J, Bronowicki JP, Pereira IA, Mougenel JL, Faou AE: **Sulfate-reducing bacteria in human feces and their association with inflammatory bowel diseases**. *FEMS Microbiol Ecol* 2002, **40**(2):107-112.
32. Oliphant K, Allen-Vercoe E: **Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health**. *Microbiome* 2019, **7**(1):91.
33. Pinto E, Anselmo M, Calha M, Bottrill A, Duarte I, Andrew PW, Faleiro ML: **The intestinal proteome of diabetic and control children is enriched with different microbial and host proteins**. *Microbiology* 2017, **163**(2):161-174.
34. Salyers AA, West SE, Vercellotti JR, Wilkins TD: **Fermentation of mucins and plant polysaccharides by anaerobic bacteria from the human colon**. *Appl Environ Microbiol* 1977, **34**(5):529-533.
35. Pamela McInnes MC: **Manual of Procedures for Human Microbiome Project Core Microbiome SamplingProtocol A HMP Protocol # 07-001**. 2010(Version Number: 12.0).
36. Macfarlane GT, Macfarlane S, Gibson GR: **Validation of a Three-Stage Compound Continuous Culture System for Investigating the Effect of Retention Time on the Ecology and Metabolism of Bacteria in the Human Colon**. *Microb Ecol* 1998, **35**(2):180-187.
37. Apprill A MS, Parsons R, Weber L: **Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton**. *Aquatic Microbial Ecology* 2015, **75**:129-137.
38. Parada AE, Needham DM, Fuhrman JA: **Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples**. *Environ Microbiol* 2016, **18**(5):1403-1414.
39. Gohl DM, Vangay P, Garbe J, MacLean A, Hauge A, Becker A, Gould TJ, Clayton JB, Johnson TJ, Hunter R *et al*: **Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies**. *Nat Biotechnol* 2016, **34**(9):942-949.
40. Martin M: **Cutadapt removes adapter sequences from high-throughput sequencing reads**. *EMBnetjournal* 2011, **17**(1).
41. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F *et al*: **Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2**. *Nat Biotechnol* 2019, **37**(8):852-857.
42. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP: **DADA2: High-resolution sample inference from Illumina amplicon data**. *Nat Methods* 2016, **13**(7):581-583.
43. Price MN, Dehal PS, Arkin AP: **FastTree 2–approximately maximum-likelihood trees for large alignments**. *PLoS One* 2010, **5**(3):e9490.
44. Uritskiy GV, DiRuggiero J, Taylor J: **MetaWRAP-a flexible pipeline for genome-resolved metagenomic data analysis**. *Microbiome* 2018, **6**(1):158.

45. Li D, Liu CM, Luo R, Sadakane K, Lam TW: **MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph**. *Bioinformatics* 2015, **31**(10):1674-1676.
46. Li D, Luo R, Liu CM, Leung CM, Ting HF, Sadakane K, Yamashita H, Lam TW: **MEGAHIT v1.0: A fast and scalable metagenome assembler driven by advanced methodologies and community practices**. *Methods* 2016, **102**:3-11.
47. Kang DD, Li F, Kirton E, Thomas A, Egan R, An H, Wang Z: **MetaBAT 2: an adaptive binning algorithm for robust and efficient genome reconstruction from metagenome assemblies**. *PeerJ* 2019, **7**:e7359.
48. Wu YW, Simmons BA, Singer SW: **MaxBin 2.0: an automated binning algorithm to recover genomes from multiple metagenomic datasets**. *Bioinformatics* 2016, **32**(4):605-607.
49. Alneberg J, Bjarnason BS, de Bruijn I, Schirmer M, Quick J, Ijaz UZ, Lahti L, Loman NJ, Andersson AF, Quince C: **Binning metagenomic contigs by coverage and composition**. *Nat Methods* 2014, **11**(11):1144-1146.
50. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C: **Salmon provides fast and bias-aware quantification of transcript expression**. *Nat Methods* 2017, **14**(4):417-419.
51. Seemann T: **Prokka: rapid prokaryotic genome annotation**. *Bioinformatics* 2014, **30**(14):2068-2069.
52. Huang L, Zhang H, Wu P, Entwistle S, Li X, Yohe T, Yi H, Yang Z, Yin Y: **dbCAN-seq: a database of carbohydrate-active enzyme (CAZyme) sequence and annotation**. *Nucleic Acids Res* 2018, **46**(D1):D516-D521.
53. Zhang H, Yohe T, Huang L, Entwistle S, Wu P, Yang Z, Busk PK, Xu Y, Yin Y: **dbCAN2: a meta server for automated carbohydrate-active enzyme annotation**. *Nucleic Acids Res* 2018, **46**(W1):W95-W101.
54. Parks DH, Chuvochina M, Waite DW, Rinke C, Skarshewski A, Chaumeil PA, Hugenholtz P: **A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life**. *Nat Biotechnol* 2018.
55. Eren AM, Esen OC, Quince C, Vineis JH, Morrison HG, Sogin ML, Delmont TO: **Anvi'o: an advanced analysis and visualization platform for 'omics data**. *PeerJ* 2015, **3**:e1319.
56. Truong DT, Franzosa EA, Tickle TL, Scholz M, Weingart G, Pasolli E, Tett A, Huttenhower C, Segata N: **MetaPhlAn2 for enhanced metagenomic taxonomic profiling**. *Nat Methods* 2015, **12**(10):902-903.
57. Weinstock GM: **Genomic approaches to studying the human microbiota**. *Nature* 2012, **489**(7415):250-256.
58. Stefanini I, Cavalieri D: **Metagenomic Approaches to Investigate the Contribution of the Vineyard Environment to the Quality of Wine Fermentation: Potentials and Difficulties**. *Front Microbiol* 2018, **9**:991.
59. R Core Team: **R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.** In.; 2015.
60. Wickham H: **ggplot2: Elegant Graphics for Data Analysis**: Springer-Verlag New York; 2009.

Figures

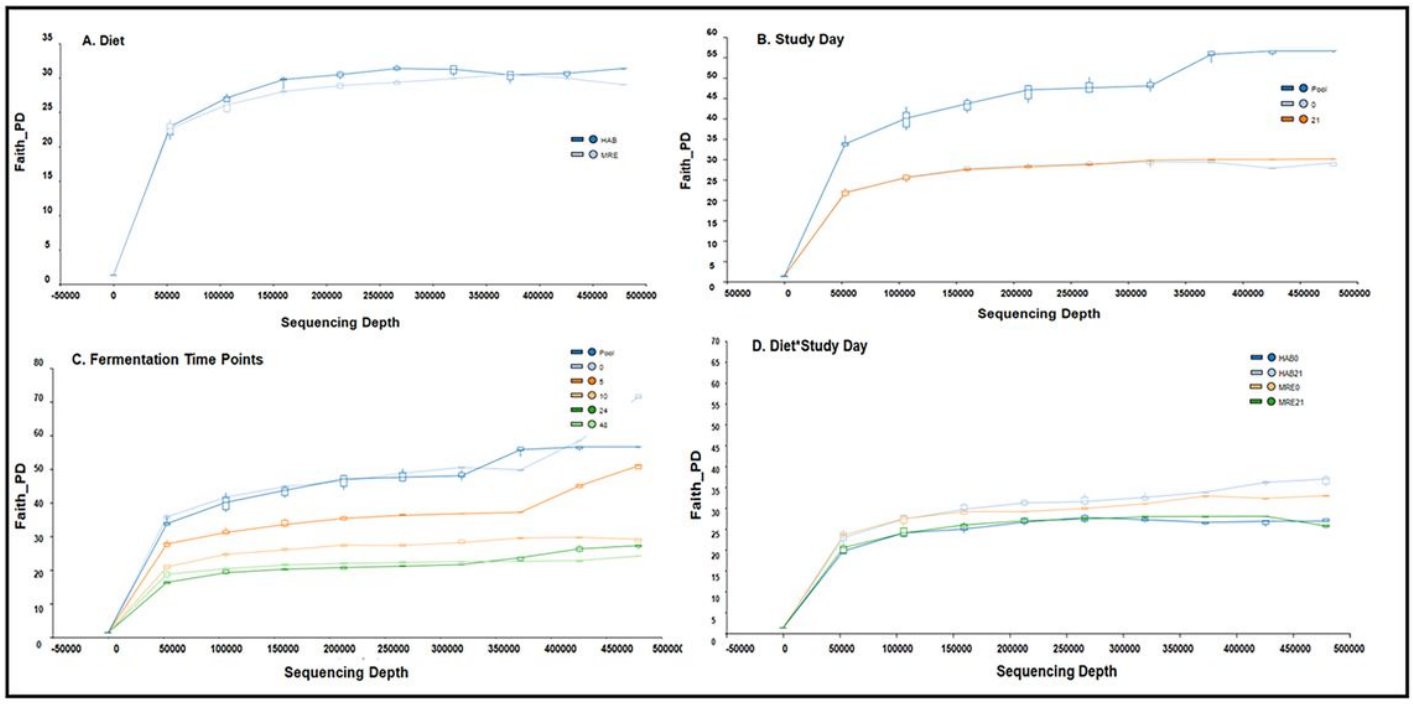

Figure 1

Alpha Diversity Plots: Faith_PD Rarefaction curves for Alpha Diversity Analysis. As the Faith Phylogenetic Diversity (Faith_PD) shows, there are no observed differences in alpha diversity by Diet (A) and Study Day (B). Alpha diversity decreases with fermentation time (C). There are no differences in the interaction Diet*Study Day (D).

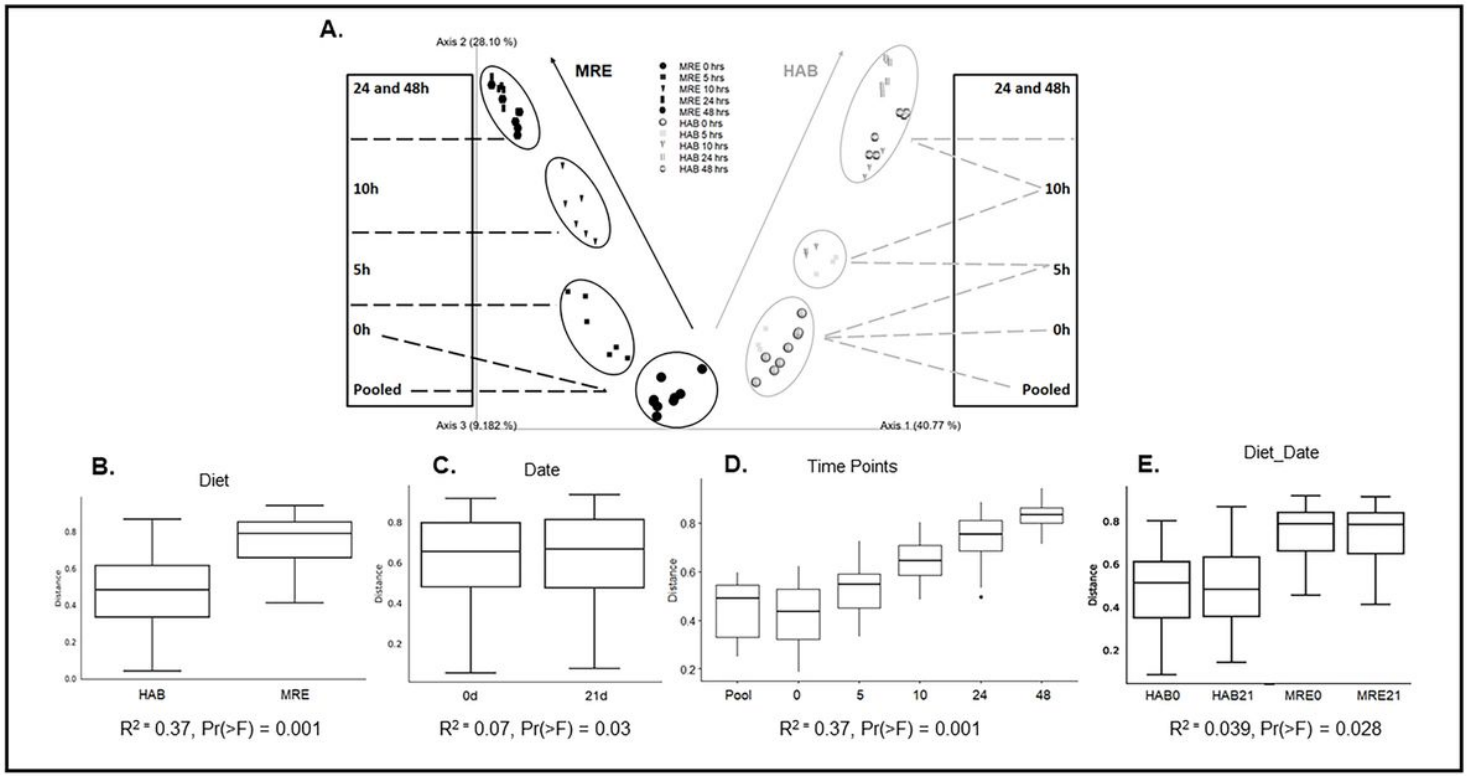

Figure 2

16S rRNA: Bray-Curtis PCoA and PERMANOVA PCoA (A) shows a divergence and clustering by Diet and Fermentation Time Points but not by Study Day (0d vs 21d). B, C, D, and E corresponds to the Bray-Curtis Distance and Adonis PERMANOVA analysis by Diet, Date, Fermentation Time Points and Diet/Date groups, respectively. P-values are for comparisons to the Pool time point.

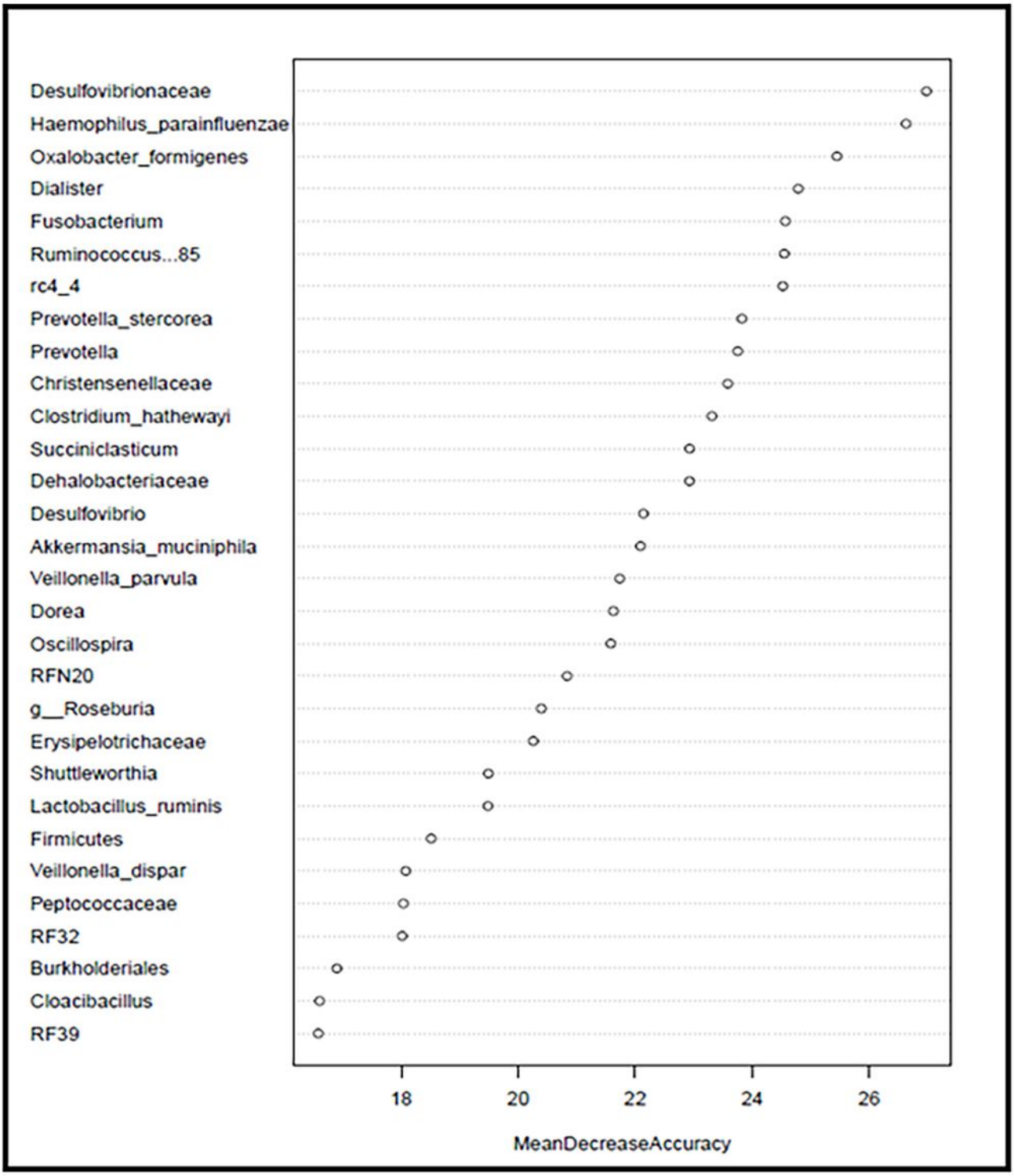

Figure 3

16S rRNA RandomForest Analysis RandomForest algorithm employed to the MRE-HAB diet groups and 0h-24h combined fermentation time points data. 30 taxa out of 201 are contributing to the combined community changes. X axis represents the Mean Decrease Accuracy for the 30 taxa. The higher the number, the more important in classifying the data.

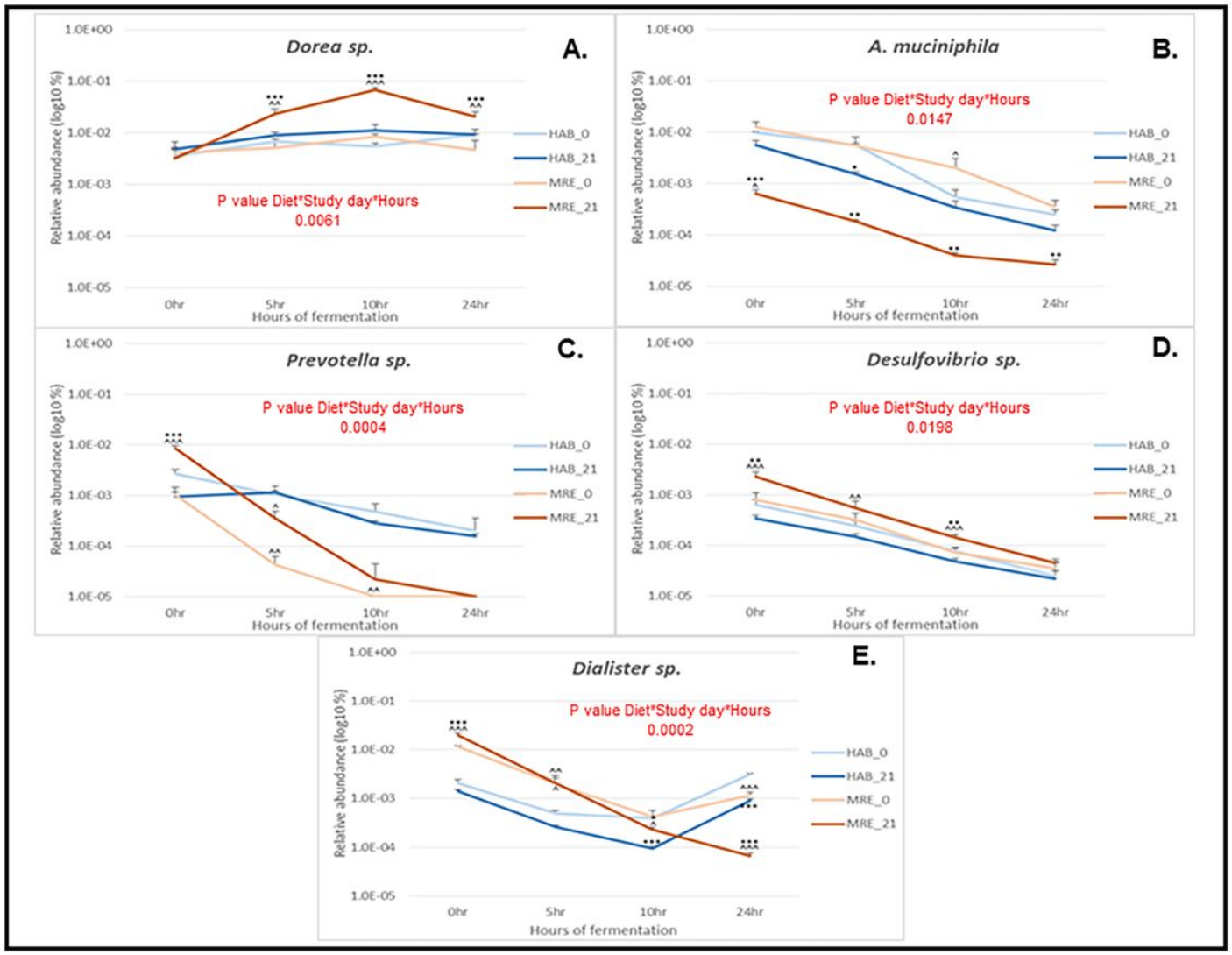

Figure 4

16S rRNA Linear Mixed Model Analysis for 5 organisms Linear mixed model analysis (multivariate ANOVA with repeated measures) for Diet*StudyDay*Fermentation Time Points interaction. Linear graphs representing the 5 organisms out of 30 that have significant 3-way interaction for the different fermentation time points and Diet/Date groups in function of their relative abundance: Dorea sp. (A), Akkermansia muciniphila (B), Prevotella sp. (C), Desulfovibrio sp, (D), and Dialister sp. (E). Exact p-values from the test are also reported in Table S1. Pairwise multiple comparison (Tukeys HSD Analysis) for the 5 organism obtained after the Linear mixed model analysis within each time-point, is represent by symbols: a (*) symbol indicates a difference between study days 0 and 21 for the same diet, and a (^) symbol indicates a difference between MRE and habitual (HAB) diets for the same study day. One symbol indicates $p \leq 0.05$, two symbols indicates $p \leq 0.01$, and three symbols indicates $p \leq 0.001$. Exact p-values from the test are also reported in Table S2.

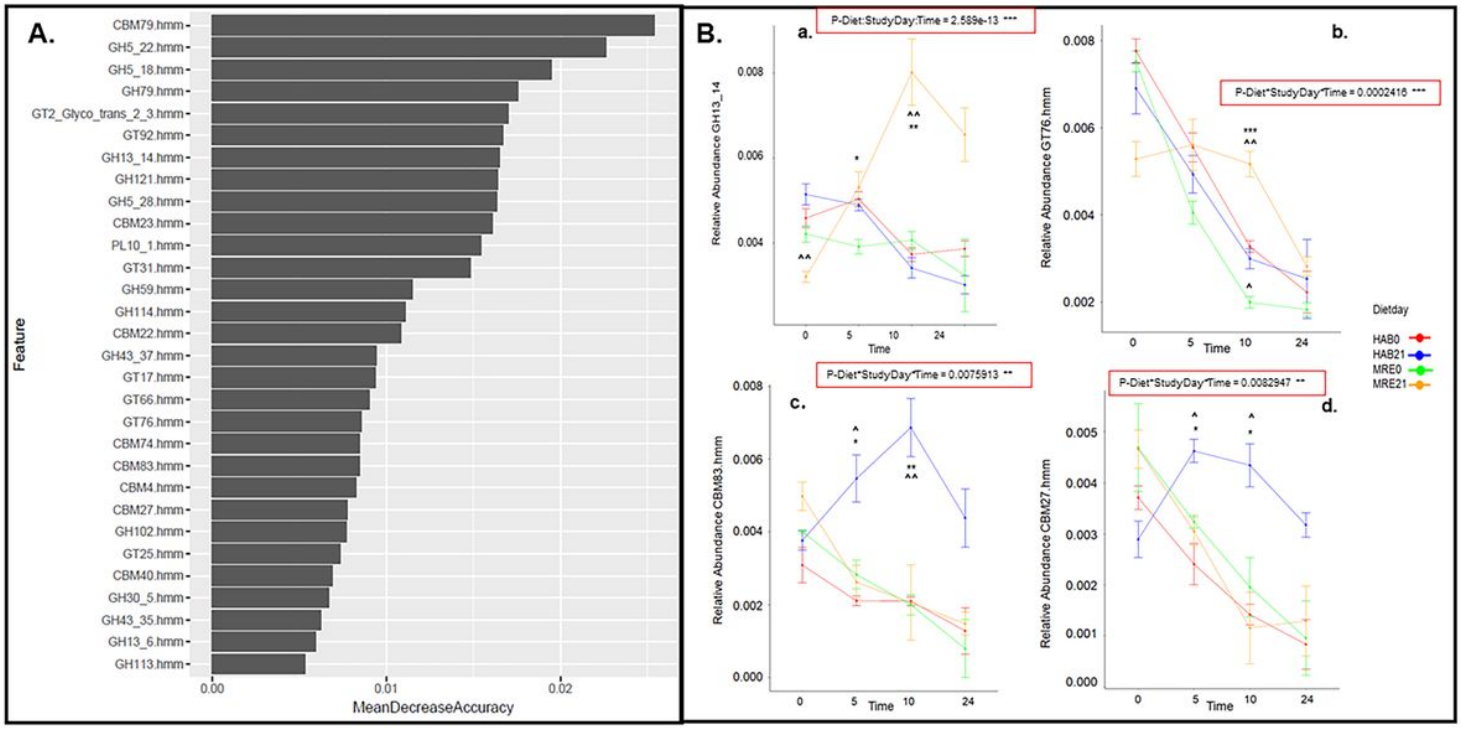

Figure 5

Cazymes RandomForest Analysis (A) and CAZymes Relative Abundances by Groups and Fermentation Time Points (B) RandomForest Analysis that shows the 30 CAZymes that are changing across all the sample groups (A). The algorithm was employed to the MRE-HAB diet groups and 0h-24h fermentation time points combined data. Linear mixed model analysis (multivariate ANOVA with repeated measures) for Diet*StudyDay*Fermentation Time Points interaction (B). Linear graphs representing the 4 CAZymes out of 30 that have significant 3-way interaction for the different fermentation time points and Diet/Date groups in function of their relative abundance: GH13_14 (a), GT76 (b), CBM83 (c) and CMB27 (d). (*) symbol indicates a difference between study days 0 and 21 for the same diet, and a (^) symbol indicates a difference between MRE and habitual (HAB) diets for the same study day. One symbol indicates $p \leq 0.05$, two symbols indicates $p \leq 0.01$, and three symbols indicates $p \leq 0.001$.

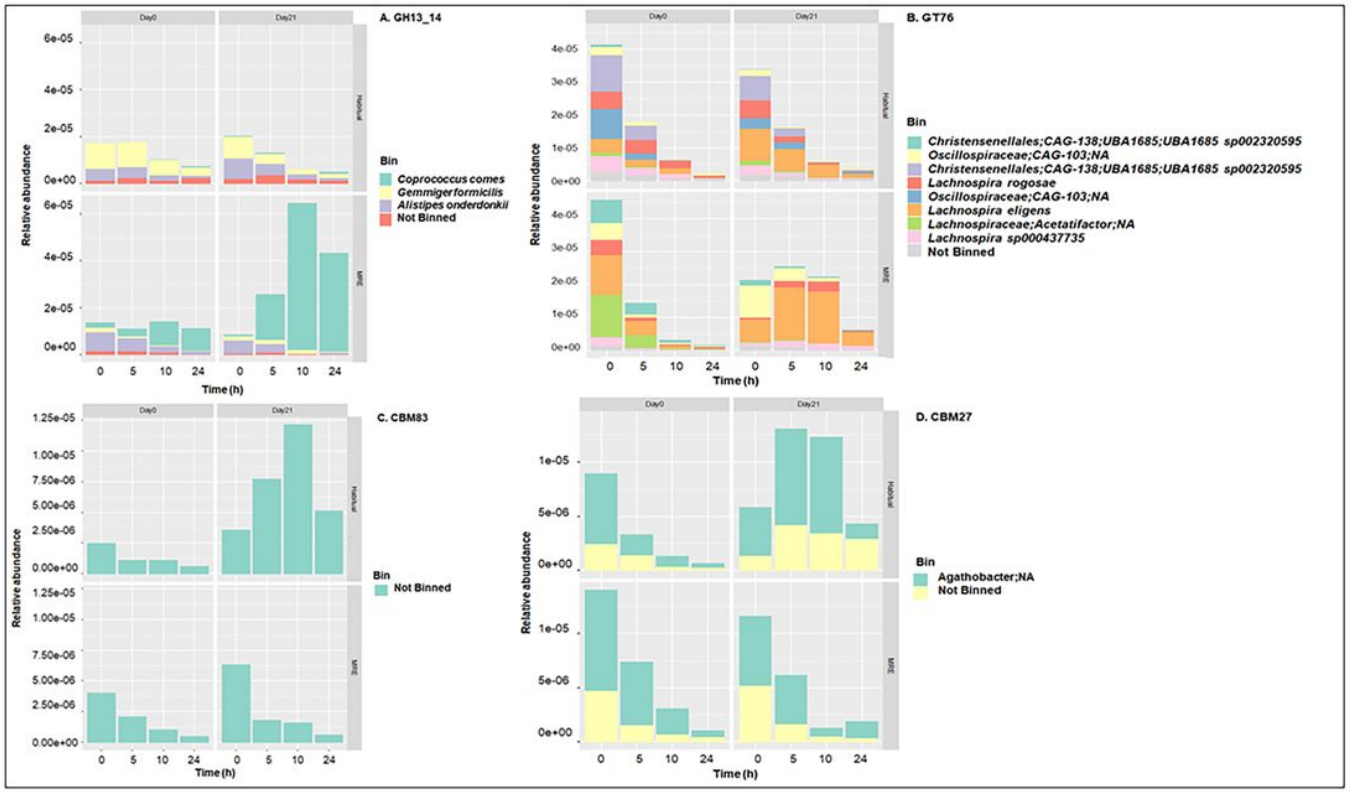

Figure 6

CAZyme's Bar Plot by Species Bar graphs linking CAZymes relative abundances with bacterial species bins. (A) corresponds to GH13_14, (B) GT76, (C) CBM83 and (D) to CMB27. MAG and taxonomic breakdown of GH13_14 by Diet*Date indicated that the increased abundance in MRE Day 21 samples was due to a Coproccocus comes MAG (A) and CAZyme GT76 and its prevalence in the MRE day 21 group associated with Lachnospira eligens (B).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SCHEME1Schematicrepresentationoftheinvitrofermentationprotocol300PPI1.jpg
- RSSupplementaryMay72021.ppt