Analysis of Gut Microbiome Dynamics Following Prebiotic Supplementation

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Abstract. The human gut microbiota is a complex and dynamic ecosystem, whose composition and functionality are modulated by components such as prebiotics. This project aims to investigate how prebiotic supplementation alters microbial composition and to uncover the metabolomic basis behind the enrichment of specific bacterial taxa, focusing on their enzymatic capacity to metabolize prebiotics. We developed a modular Python-based bioinformatics workflow to integrate 16S rRNA metataxonomic data with functional prediction. The workflow includes normalization of abundance data based on 16S gene copy numbers, statistical selection of relevant taxa using MicrobiomeAnalyst outputs, and functional annotation through enzyme commission (EC) numbers and KEGG pathways. This pipeline enables the identification of microorganisms enriched after treatment and their potential to metabolize specific prebiotics. This tool bridges the gap between taxonomy and function and provides a scalable framework for microbiome-prebiotic interaction studies.

Keywords: gut microbiota, statistical analysis, KEGG pathways, functional characterization, bioinformatics pipeline

1 State of the Art

Human gut microbiota is a complex ecosystem of trillions of microorganisms that play essential roles in digestion, immunity, and overall health [1]. Prebiotics, defined as substrates thar are selectively utilized by host microorganisms conferring a health benefit, are known to influence the growth of beneficial bacteria [2-4]. Recent advances have shown that the specific response of gut microbiota to prebiotics is dependent on their genomic capability to metabolize these compounds [5].

Understanding how gut microbiota metabolize prebiotics requires not only biological insight but also the application of bioinformatics tools capable of interpreting large-scale sequencing data. Advancements in high-throughput sequencing, especially 16S rRNA gene sequencing, have allowed researchers to profile microbial communities at unprecedented depth. The 16S rRNA gene, highly conserved across bacteria but with hypervariable regions, is commonly used for bacterial identifications and phylogenetic studies. This gene contains nine hypervariable regions (V1-V9) interspersed with conserved sequences and amplifying and sequencing specific variable regions allows researchers to classify bacteria to the genus or species level [6]. When using Illumina

sequencing, a method of high-throughput sequencing technology, microbial DNA is first extracted from samples such as fecal material, to then amplify a target region, commonly V3-V4, of the 16S rRNA gene using specific primers [7]. Then, the amplicons are barcoded, prepared for sequencing and the Illumina MiSeq generates millions of short reads, 150-300 bp paired-end reads, which originates a text file that contains the raw DNA sequence reads (FASTQ file) and corresponding quality scores (Phred scores) used for downstream analysis, which indicate the confidence in each base call [8,9]. These files form the starting point of any microbiome bioinformatic workflow.

The FASTQ files are processed to quality filter, trim reads to remove low-quality bases, merge paired end reads and de-noise to distinguish Amplicon Sequence Variants (ASVs) or cluster Operational Taxonomic Units (OTUs). For this, two widely used command-line bioinformatics platforms are used: QIIME2 and mothur. QIIME2 offers modular plugins for each step: importing data, denoising, taxonomic assignment, diversity analysis, and visualization [10,11]. Meanwhile, mothur is another powerful pipeline, particularly strong for OUT-based workflows, also performed via the command line with a sequence of commands for each processing step [11,12]. The processed sequences are then matched to known bacterial sequences in reference databases such as greengenes which is a commonly used database for 16S rRNA gene sequences and SILVA and RDP which are alternatives with broader or more updated coverage [13-15]. Moreover, to analyze and interpret the large datasets generated in microbiome studies, MicrobiomeAnalyst enables statistical analysis, visualization, and interpretation of microbiome data, making it accessible for researchers with varying levels of bioinformatics expertise [16].

However, 16S rRNA sequencing only provides taxonomic insight and lacks direct insight into microbial function and to address this, functional inference tools like PICRUSt2 that predict the gene content of microbial communities based on taxonomic composition, helping infer the metabolic pathway that are likely present in a microbiome [17]. For example, taxa such as *Bifidobacterium* and certain *Bacteroides* species often increase after prebiotic intake, such as inulin or fructooligosaccharides (FOS), due to their ability to encode glycoside hydrolases and other carbohydrate-active enzymes (CAZymes) [18,19]. Mining these functional traits can reveal mechanisms driving microbiota shifts in response to prebiotic interventions.

Despite these advancements, a gap remains in linking statistically significant taxonomic changes, especially those occurring under specific prebiotic treatments, with their precise enzymatic potential. This limitation is particularly evident when 16S-based analyses do not explicitly connect abundance shifts to metabolic capabilities. To fill this gap, we developed a modular bioinformatics pipeline implemented in Python which performs the normalization of abundance data based on 16S rRNA gene copy numbers for each taxon, thereby correcting potential biases introduced by differential gene copy numbers across bacteria. Next, statistical outputs from MicrobiomeAnalyst, such as, p-value, LDA (Linear Discriminant Analysis) score, mean relative abundance, and core microbiome presence, will be used to select taxa of biological relevance. Then, the workflow will retrieve enzyme commission (EC) numbers associated with the selected organisms through KEGG (Kyoto Encyclopedia of Genes and Genomes) queries, which will then be systematically linked to the ECs of pre-defined KEGG metabolic

pathways of interest, creating a compatibility matrix which will allow researchers to identify not only the taxa that increases under prebiotic influence, but also why, based on their enzymatic potential to participate in key metabolic processes.

2 Aims

In this project, we aim to analyze how prebiotic supplementation affects microbiota composition and explore why certain bacterial taxa thrive by investigating their genomic potential, particularly their ability to produce enzymes that metabolize specific prebiotics. Thus, the main aims of this project are to conduct a bioinformatic survey linking taxonomic shifts to functional traits in gut microbiota communities.

In order to achieve this goal, a modular workflow that normalizes abundance data using 16S copy numbers, uses MicrobiomeAnalyst outputs and selects taxa based on LDA scores, p-values, relative abundance, and core microbiome status, extracts functional gene information from taxa and relevant KEGG pathways to finally construct a compatibility table that reveals the role each bacterium had in prebiotic degradation pathways, was established.

3 Preliminary Problem Analysis

Despite advancements in sequencing and bioinformatics, a gap remains between identifying which bacteria grow in response to prebiotics and understanding why these taxa are favored. Most workflows stop at reporting taxa that increase post-treatment, without mechanistically explaining why these bacteria become enriched. This project addresses that gap by linking bioinformatic predictions (e.g., EC numbers) to actual pathway-level interpretations.

Furthermore, 16S rRNA gene sequencing offers limited resolution, as it identifies bacteria only at the genus or species level and does not directly inform functional genes. Tools like PICTUSt2 infer function but require careful validation and integration with robust statistical selection criteria [17]. GUMPP is another tool that unifies the taxonomic and functional prediction process in a reproducible and modular fashion, reducing variability and manual overhear in the analysis [20]. Nevertheless, neither of these tools explicitly correlates taxa abundance changes with the presence of an specific metabolic enzyme relevant to the prebiotic being studied.

By incorporating copy number normalization, differential abundance analysis, and EC-pathway mapping, our pipeline enables this final and essential link by constructing a compatibility matrix of bacteria * ECs * pathways and by identifying taxa capable of fully or partially degrading prebiotics. This unified approach bridges microbiota composition with predicted metabolic activity, enabling researchers to move beyond correlation and into casual functional interpretation.

4 Methodology

This project proposes a modular bioinformatics pipeline implemented in Python that integrates taxonomic and functional analysis of gut microbiota communities in response to prebiotic supplementation. The pipeline comprises three main phases:

- Phase 1: Normalization of taxonomic abundance data using 16S rRNA gene copy numbers.
- Phase 2: Selection of microorganisms of interest based on 16S rRNA gene sequencing and statistical analysis.
- Phase 3: Functional inference and enzymatic mapping using KEGG and EC annotations.

The workflow allows users to start from pre-analyzed abundance tables and incorporates public databases and tools such as MicrobiomeAnalyst, rrnDB and KEGG REST API. The Python scripts described below can be found in: https://github.com/VaneBR/Project/tree/main.

4.1 Phase 1: Normalization

The first step, before performing statistical comparisons, is to normalize the relative abundance of each taxon based on 16S rRNA gene copy number (Figure 1). By normalizing this data, we ensure that taxa with high copy numbers are not overrepresented due to technical biases in amplification.

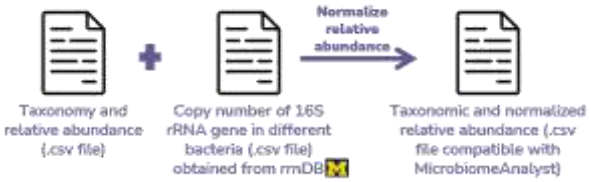


Fig. 1. Representation of the workflow for normalization of relative abundance using 16S copy number (Phase 1). The input consists of a .csv file that contains taxonomy and relative abundance obtained from 16S rRNA gene sequencing and a reference file with the copy number of several taxa obtained from the database rrnDB. The output is a MicrobiomeAnalyst-compatible .csv file that contains taxonomy and normalized abundance.

The input is a .csv file obtained from 16S rRNA gene sequencing, that contains taxonomy and relative abundance and a reference file with known 16S copy numbers per taxon obtained from the database rrnDB (Ribosomal RNA Operon Copy Number Database) [21]. Therefore, the custom Python script, **normalize_16s.py**, first identifies the best-matching taxonomic level from each row of the data file to the reference copy number file, the divide raw abundance values by the corresponding copy number (Equation 1) and finally formats the resulting file for compatibility with MicrobiomeAnalyst input. Thus, the output is a normalized abundance file (**matrix_normalized_microbiomeanalyst.csv**) used in the next statistical analysis phase.

$$Normalized\ abundance = \frac{Relative\ abundance}{Copy\ number\ of\ 16S\ rRNA\ gene} \quad (1)$$

4.2 Phase 2: Selection of Microorganisms of Interest

The input for Phase 2 is a normalized 16S rRNA taxonomic profile of gut microbiota, formatted for compatibility with MicrobiomeAnalyst which is a tool used to perform statistical comparisons and calculate metrics such as p-value, LDA score, relative abundance, and core microbiome presence [16]. The p-value indicates the statistically significant difference in taxon abundance between groups, while LDA score reflects the effect size and discriminative power of each taxon in separating conditions. Moreover, relative abundance quantifies how prevalent each taxon is within the microbial community and the core microbiome presence identifies taxa thar are consistently present across most samples. Thus, by selecting taxa that follows these four metrics we have a list of taxa that are statistically different and are likely to be functionally meaningful, making them strong candidates as indicators of microbial response to the prebiotic treatment.

The Python script **selection_bacterial_taxa.py**, then merges the outputs into a single table (bacterial_analysis_summary.csv) and filters taxa that meet user-defined criteria. The output is a refined list of taxa enriched and consistently abundant under specific prebiotic conditions (**selected_taxa.csv**) (Figure 2). Moreover, the script also outputs a file that contains the taxa that did not meet at least one criterion (**excluded_taxa.csv**), which the user can then explore and modify if desired to include taxa for further analysis. This list forms the foundation for functional exploration in Phase 3.

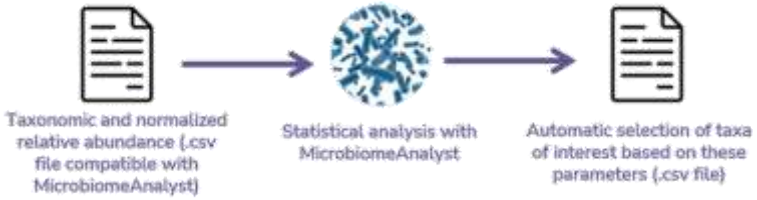


Fig. 2. Representation of the workflow for selecting microbial taxa that significantly changes in abundance following prebiotic intervention (Phase 2). The input could consist of a normalized 16S rRNA taxonomic profile MicrobiomeAnalyst-compatible file. This data as well as a metadata file the user must create, are introduced into MicrobiomeAnalyst to perform statistical analysis, diversity assessments, and visualization of taxonomic shifts. The output is a list of microbial taxa that significantly increase in abundance compared to baseline and control conditions or remain abundant over time.

4.3 Phase 3: Functional Analysis

This phase aims to functionally characterize the selected bacterial taxa by determining their enzymatic capabilities and identifying their role in the metabolism of specific prebiotics and relevant pathways (Figure 3), such as those linked to short-chain fatty acid production SCFAs (e.g., butyrate) and key metabolic intermediates such as acetyl-CoA.

Each taxon's functional profile is retrieved via the KEGG REST API, using the genus and species name to extract the corresponding KEGG organism code and associated EC numbers. KEGG is a comprehensive database resource that integrates genomic,

chemical, and functional information [22]; thus, it is widely used to understand biological pathways, metabolic networks, and molecular functions. In this project KEGG is leveraged to link bacterial taxa to specific EC numbers and to map those enzymes to annotated metabolic pathways, making it possible to interpret which taxa can carry out or contribute to the degradation of metabolites.

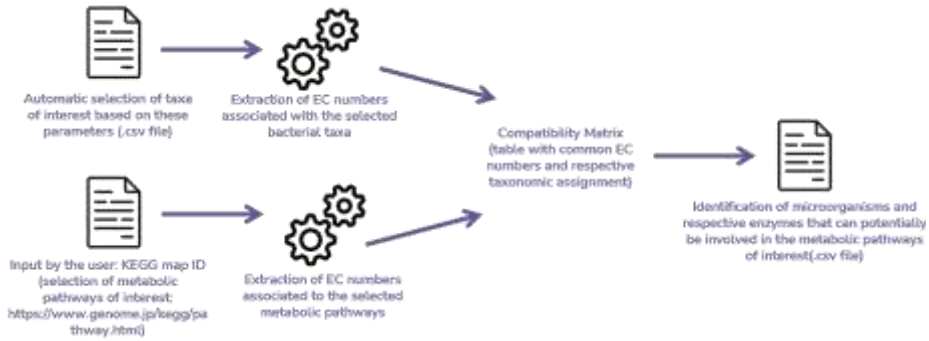


Fig. 3. Illustration of the functional analysis workflow to determine the metabolic potential of the selected microorganisms (Phase 3). The input consists of a list of microbial taxa separated by Genus and Species obtained from Phase 2. The analysis includes the extraction of EC numbers associated with the selected bacterial taxa and metabolic pathways using KEGG Ortholog annotations. The final output is a comprehensive functional characterization linking microbial taxa to relevant metabolic pathways.

These EC numbers are then cross-referenced with a user-provided list of KEGG IDs metabolic pathways; thus, a compatibility matrix that maps bacteria enzymes and their corresponding metabolic pathways is constructed. The Python module **functional_information.py** is used to match EC numbers of taxa to pathways and output a structured table (**compatibility_table_with _names.csv**) for analysis. This allows researchers to identify which taxa encode complete or partial enzyme sets for a given pathway and the potential for cooperative degradation.

This methodology creates a functional bridge between taxonomic enrichment and enzymatic potential, offering a biologically grounded interpretation of gur microbiota responses to dietary interventions.

5 Results & Discussion

A cellulose case study was analyzed using this Python-based workflow. In this case study we had 3 different conditions: baseline, negative control (without cellulose) and treatment with cellulose, in 5 different types of donors (A, B, C, D and E) and in two different timepoints 0h and 24h.

After normalizing relative abundance of the taxonomic taxa obtained from 16S rRNA gene sequencing, 27 relevant taxa were selected using MicrobiomeAnalyst (Figure 1). These taxa were identified as being differentially abundant across experimental conditions, based on the combination of statistical metrics mentioned above, thus these

taxa represent a group of key microbial responders to the cellulose treatment which significantly increased after cellulose exposure, when compared to the negative control and have a strong discriminative power.

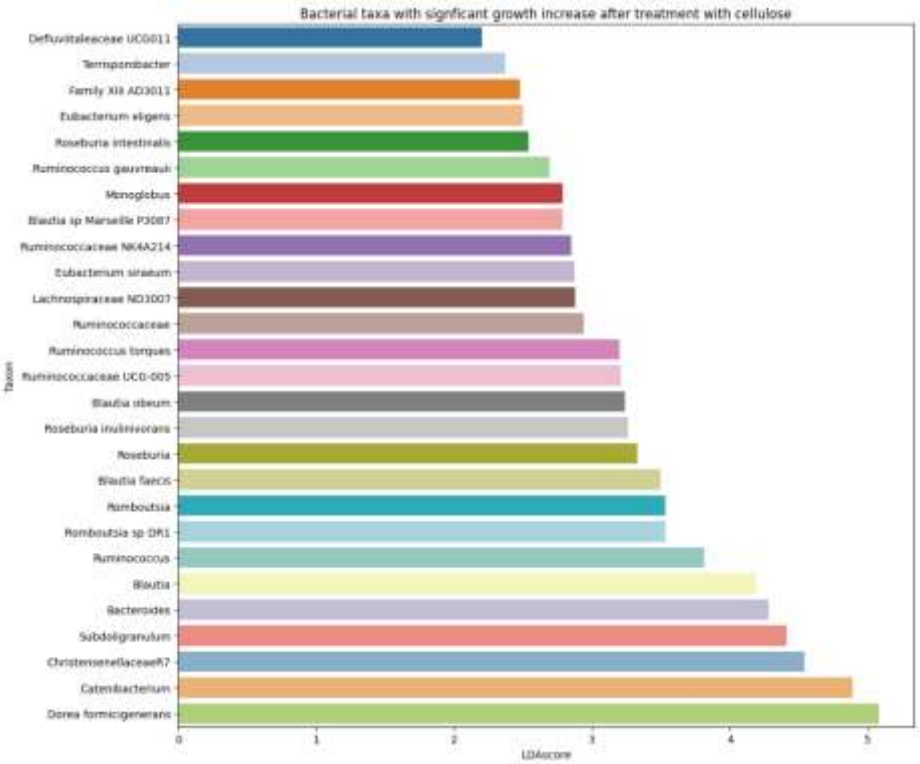


Figure 4. Linear Discriminant Analysis (LDA) scores of the 27 bacterial taxa selected which show to have significantly enriched after cellulose treatment compared to the negative control, Bars represent taxa with increased relative abundance in the cellulose treatment group since high LDA scores reflect strong associations with the treatment condition. The values used to create the bar-plot were taken from MicrobiomeAnalyst.

To perform the functional analysis, we selected 12 metabolic pathways (Table 1) which include pathways that are upregulated in response to cellulose treatment, are relevant in gut microbiota (such as SCFA production) and are core to microbial metabolism (such as fermentation and carbohydrate metabolism).

Table 1. Selected metabolic pathways with its respective KEGG map ID and relevance.

Metabolic Pathway	KEGG map ID	Function / Importance
Starch and sucrose metabolism	map00500	Key for digesting dietary carbohydrates and production of glucose
Amino sugar and nucleotide sugar metabolism	map00520	Involved in cell wall biosynthesis and host-microbe interactions
Pentose and glucuronate interconversions	map00040	Processes sugars from dietary fibers
Pentose phosphate pathway	map00030	Generates NADPH and pentose
Glycolysis / Gluconeogenesis	map00010	Central pathway for energy production and glucose synthesis
Citrate cycle (TCA cycle)	map00020	Core of aerobic respiration
Pyruvate metabolism	map00620	Links glycolysis, fermentation, and the TCA cycle
Propanoate metabolism	map00640	Leads to the production of propionate
Butanoate metabolism	map00650	Produces butyrate
Glycosaminoglycan degradation	map00531	Breaks down host-derived glycosaminoglycans
Fatty acid biosynthesis	map00061	Converts acetyl-CoA to fatty acids

After performing the functional analysis, we had a compatibility matrix with 2785 matches which were analyzed using the interactive table (**interactive_table.py**) and after observing the enzymes found in both the selected bacteria taxa and metabolic pathways, we arrived at a possible pathway for degrading and utilizing cellulose with the enzymes present in both the bacteria and the selected metabolic pathway (Figure 5). This hypothesized pathway can be divided into 4 main stages:

- Stage 1: Cellulose degradation and monosaccharide release
- Stage 2: Glucose activation and entry into Glycolysis
- Stage 3: Glycolysis to pyruvate
- Stage 4: SCFAs and fermentation products
- Stage 5: Fatty acid biosynthesis (from acetyl-CoA)

Stage 1 represents cellulose degradation which essentially involves the action of two enzymes, cellulase and beta-glucosidase, which help transform cellulose into smaller sugars such as glucose, making carbon sources accessible for microbial metabolism and the start of stage 2. Stage 2 and stage 3 serve as the core energy-yielding pathway since the enzymatic conversion of glucose into pyruvate generates ATP and reduces equivalents. Stage 4 metabolizes pyruvate intro short-chain fatty acids (SCFAs) like acetate, lactate and ethanol, which are the key end-products in anaerobic microbial fermentation. In stage 5, acetyl-CoA intermediate is redirected toward fatty acid biosynthesis, which is an anabolic process that elongates carbon chains step by step, producing long-chain fatty acids. This pathway links central carbon metabolism to lipid biosynthesis which reflects a metabolic shift from energy generation to biomass production.

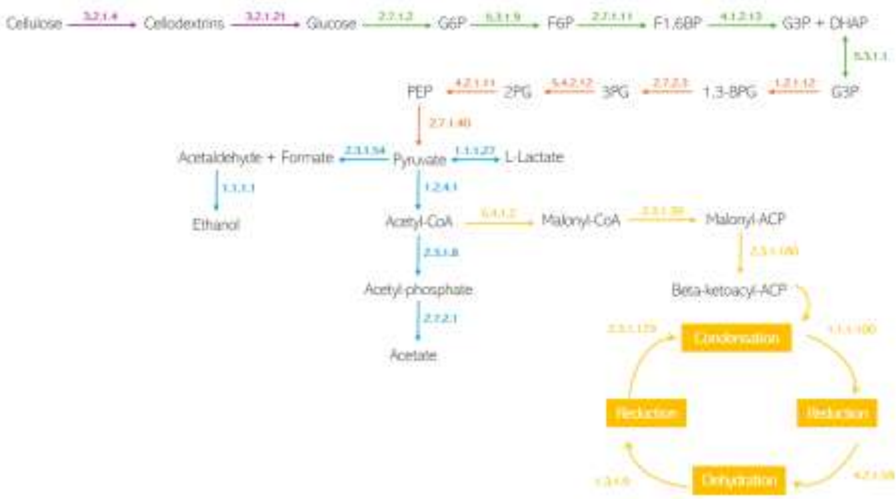
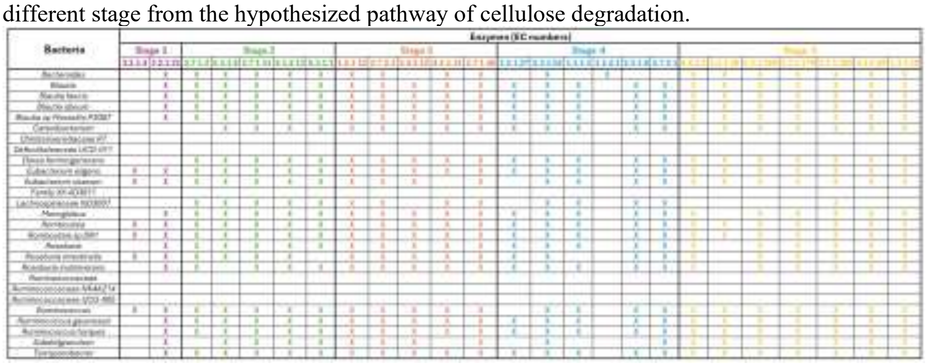


Fig 5. Hypothesized pathway of cellulose degradation and conversion into glucose, pyruvate, SCFAs and fermentation products. This pathway was designed using the enzymes found in the compatibility matrix table that contains the enzymes that are present in both the selected relevant bacteria taxa and metabolic pathways of interest. The pathway is divided into 5 stages: Stage 1 (Cellulose degradation and monosaccharide release), Stage 2 (Glucose activation and entry into Glycolysis), Stage 3 (Glycolysis to pyruvate), Stage 4 (SCFAs and fermentation products) and Stage 5 (Fatty acid biosynthesis from acetyl-CoA)

Table 2 represents the 27 bacterial taxa selected and which enzymes from each stage do they express. The bacteria that have both enzymes in stage 1 are the primary bacteria for cellulose degradation, without these bacteria and these enzymes, cellulose could not be utilized. Of the 27 selected bacteria, only 6 are responsible for the degradation of cellulose and its conversion into glucose (stage 1). Then 17 bacteria have all 5 enzymes necessary for stage 2 and for stage 3 there were found 17 bacteria that expressed all 5 enzymes necessary. In the first 3 stages, only 5 bacteria have all the enzymes necessary: *Eubacterium eligens*, *Romboutsia*, *Ruminococcus*, *Rosemburia intestinalis* and *Romboutsia sp DR1*.

Table 2. Representation of the different enzymes the 27 selected bacterial taxa have in each different stage from the hypothesized pathway of cellulose degradation.



Several different bacteria express enzymes necessary for stage 4, with 16 bacteria containing the enzyme responsible for lactate production and 18 bacteria with all the necessary enzymes for ethanol production. Specifically, to produce acetate, only 1 bacterium has an enzyme that degrades acetyl-CoA and then other 19 bacteria continue this process with both the necessary enzymes to form acetate. Finally, for stage 5, 14 bacteria have all the enzymes necessary to complete this stage and form fatty acids. These highlights the fact that cross-feeding occurs within the microbial community, showing how metabolites produced by certain species can serve as substrate for others, fostering trophic interactions and a cooperative metabolic network.

From Table 2, we can also observe that 6 bacteria taxa (*Catenibacterium*, *Defluviitaleaceae UCG-011*, *Ruminococcaceae*, *Family XII AD3011*, *Ruminococcaceae UCG-005* and *Ruminococcaceae NK4A214*) do not have any of the enzymes involved in the hypothesized pathway for cellulose degradation. A study model study in the induction of Alzheimer's with alcohol treatment showed an increase of *Defluviitaleaceae UCG-011* in the gut microbiome of mice [23]. Moreover, a study showed that when administrating hay based on starchy ingredients to calves, there was a positive correlation between *Ruminococcaceae NK4A214*, *Family XII AD3011* and *Ruminococcaceae UCG-005*, and acetate levels, meaning that their abundance is associated with environments where acetate is abundant. Thus, these taxa might not produce acetate directly but benefit from its presence or possibly also rely on cross-fed metabolites [24].

6 Conclusion & Perspective Futures

In this project, we developed a modular Python-based workflow that integrates taxonomic and functional analysis of gut microbiota communities in response to prebiotic supplementation. The pipeline addresses key limitations of existing approaches by not only identifying bacterial taxa that significantly change in abundance, but also by linking these changes to predicted functional capabilities based on enzyme presence and pathway involvement.

The developed workflow provides a robust framework for exploring microbial functional potential in response to prebiotic interventions. This workflow first normalizes taxonomic abundance data using 16S rRNA gene copy numbers to improve the accuracy of abundance comparisons. It then integrates an automated statistical filtering using outputs from MicrobiomeAnalyst, to identify and select relevant taxa based on criteria such as p-value, LDA score, mean relative abundance, and core microbiome presence. Finally, it performs functional inference by mapping the selected taxa to EC numbers and linking these to user-defined metabolic pathways of interest.

This approach enables the generation of mechanistic hypotheses regarding microbial metabolism and interspecies cooperation, offering insight into why specific taxa may increase under prebiotic intervention. Moreover, the workflow produces an structured and interpretable table that facilitate data visualization and interpretation.

The functional analysis of the cellulose case study led to the reconstruction of a five-stage pathway outlining how selected gut microbiota taxa may degrade cellulose and convert it into key metabolic end-products. Of the 27 bacteria analyzed, only six were

found to encode both enzymes required for the initial degradation of cellulose into glucose, making them essential contributors to cellulose utilization. While many taxa encode enzymes that are used in later stages, such as glycolysis, fermentation and fatty acid biosynthesis, only 5 bacterial taxa possess a complete enzymatic toolkit for the first three stages, highlighting their central role in carbon flow. The analysis also revealed that cross-feeding is a fundamental aspect of microbial metabolism, with certain taxa relying on intermediate metabolites produced by other taxa and this cooperative interaction underscores the complexity and interdependence of the gut microbiota in processing dietary polysaccharides like cellulose.

This workflow can be expanded by integrating additional databases such as CAZy or MetaCyc which would allow a deeper exploration of carbohydrate-active enzymes and alternative metabolic routes. To further enrich the functional, UPIMAPI could be integrated into the workflow since this command line tool automates functional mapping using the UniProt database [24]. This integration could enable the retrieval of enzyme names, Gene Ontology (GO) terms, and pathway associations which could offer a more comprehensive biological interpretation of the predicted functions encoded by the selected taxa. Lastly, coupling the workflow with keggcharter [24], a pathway visualization tool, would enable direct rendering of taxa-specific metabolic potential on KEGG pathway maps, offering an intuitive representation of microbial functions in the gut ecosystem.

By combining statistical selection with functional prediction, this tool represents a scalable and customizable solution for researchers seeking to bridge the gap between taxonomy and function in microbiome studies.

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