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Metaproteomics as a tool to map prebiotic action

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Introduction

Microbiome function influences host health, disease states, and ecosystem performance, creating a strong need for analytical approaches that report not only “who is there” but also “what they are doing”. Metaproteomics addresses this need by identifying and quantifying microbial proteins, thereby providing a direct readout of community activity. However, implementing metaproteomics analyses can be difficult to implement because complex microbiome matrices introduce variability at nearly every step (Figure 1). Reproducible protein extraction and preparation (reduction, alkylation, digestion, and cleanup) must accommodate diverse organisms with different lysis susceptibilities. Likewise, LC-MS/MS acquisition must balance dynamic range, sensitivity, and throughput while maintaining robustness. Finally, species-resolved identification requires searching substantially larger sequence spaces than typical human proteomics, making stringent false discovery rate (FDR) control essential. In this work, we established a robust yet high-performance workflow that enabled the identification of more than 175,000 peptides and the quantification of >8,600 microbial proteins, supporting functional interpretation of microbiome responses to the tested prebiotic conditions.

Methods

Fecal material from a single donor was supplemented with 2′-fucosyllactose (2′-FL), inulin, pectin, or resistant dextrose and subjected to ex vivo fermentation using SFIR® technology (Cryptobiotix). Samples were collected at 0, 6, and 24 hours, homogenized using a BeatBox, and processed using the SP3-iST add-on and iST kits (PreOmics). For LC-MS/MS, 15 µg of peptides were injected onto a 2.1 × 150 mm C18 column (Waters) and separated on a Bruker Elute UHPLC system (250 µL/min; 45 min active gradient), coupled to a timsTOF Pro2 mass spectrometer (Bruker) operated in PASEF mode.

Raw DDA data were processed in FragPipe (v23.0) using MSFragger (v4.2). Peptide identifications were obtained against a database generated from a metagenome-assembled genome (MAG) derived from the fecal donor.

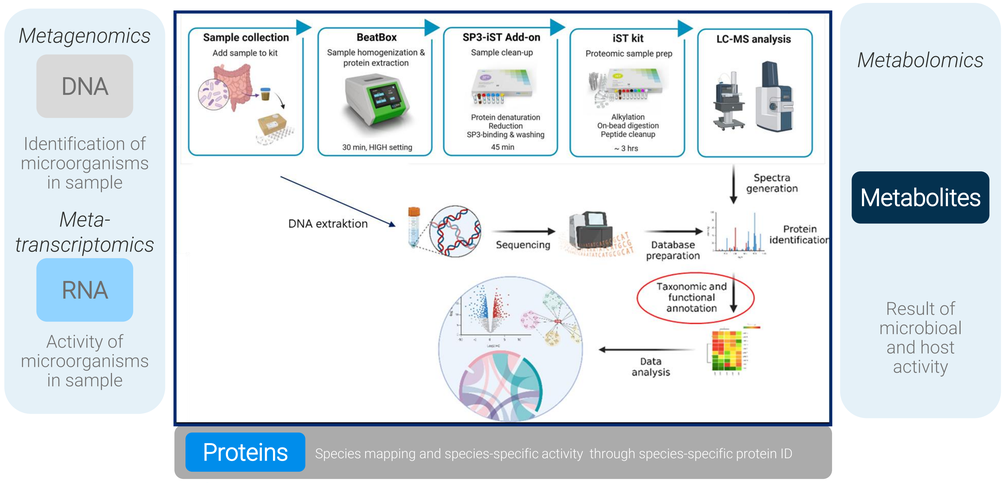


Figure 1: Experimental setup
Feces sample from one donor was spiked with 6 different compounds before a fermentation experiment followed by a Metagenomics and Metaproteomics analysis.

Results

Metaproteomics provided a direct readout of microbial activity in fecal fermentation samples (Figure 1). In total, 35,000 peptides were mapped to 14,800 proteins across 76 species. Following 2'-fucosyllactose (2'-FL) supplementation, 27 of 282 species increased 2–8× after 24 h, with total protein signal rising 3–5×.

Functional annotation confirmed activation of fucosylated carbohydrate metabolism (Figure 2), with six fucosidases showing species-specific regulation. *Blautia fusiformis* responded strongly to 2'-FL (Figure 3), while downstream enzymes including 31 fucose isomerases (two >500×) and seven fuculose-1-phosphate aldolases were upregulated, with Mediterraneibacter faecis showing >300× induction. Fifty-one beta-galactosidases were identified (Figure 2), 12 of which exceeding 500× upregulation.

Butyrate-producing pathways were activated in *Anaerobutyricum hallii*, with 32 enzymes upregulated (157–1,089×), including flavodoxin (1,089×) and lactate racemase (992×). Pectin induced CAG-274 (Table 1), resistant dextrose stimulated *Fusicatenibacter saccharivorans* (Table 2), and indole-3-lactic acid (IDA) was detected after inulin and 2'-FL supplementation (Figure 4).

Probiotic co-inoculation with 2'-FL showed strain-dependent outcomes: *Bifidobacterium breve* expanded with 64 detected proteins, while *Anaerobutyricum soehngenii* declined sharply and *Limosilactobacillus fermentum* showed no detectable protein activity.

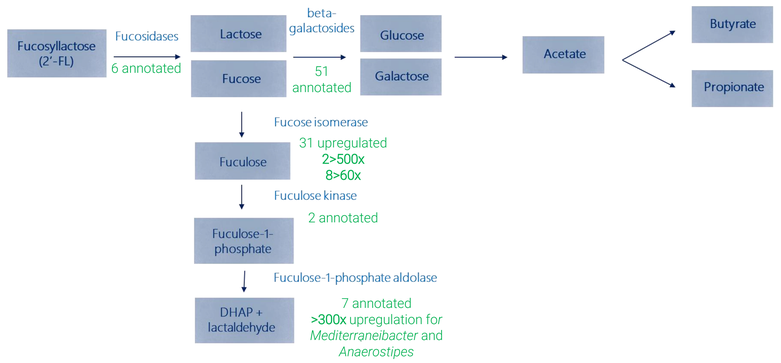
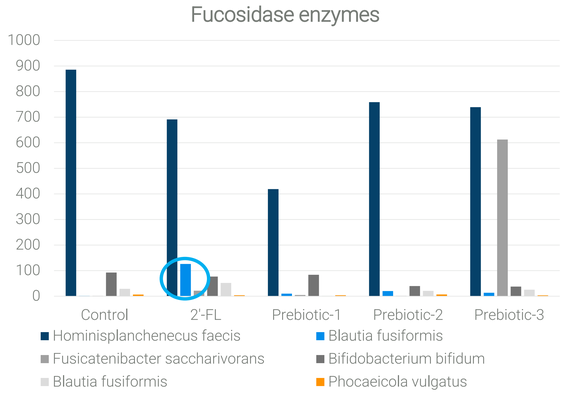


Figure 2: Fucosyllactose metabolism pathway
Fucosyllactose is found in abundance in maternal milk, and known to favor microbiotic diversity. The different pathway stages have been investigated in this study.

Figure 3: Fucosidase expression upon Prebiotic fermentation
Species-specific fucosidase expression as a function of Prebiotic fermentation.



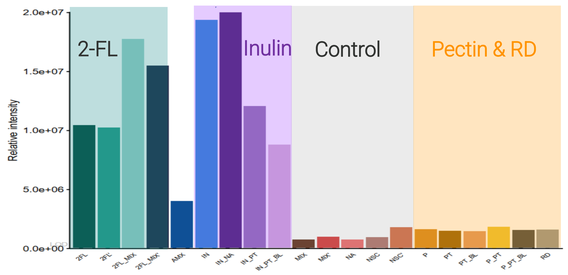


Figure 4: IDA intensity vs fermentation conditions
Measured Indole-3-lactic acid intensity as a function of fermentation conditions, highlighting a response for 2′-FL and Inulin spiked fermentations

Entry Name	Protein	Organism	Contol T0 S1-0h-NSC	Control S41-24h-NSC	Inulin S42-24h-IN	Pectin S43-24h-P	2-FL S44-24h-2FL	Resistant Dextrose S45-24h-RD
Alpha-N-arabinofuranosidase OS=Fusicatenil	727	Fusicatenibacter sacchari	1	4	31	1	1	4517
Maltose ABC transporter periplasmic protein	450	Fusicatenibacter sacchari	1	9	1	18	37	3837
DUF1533 domain-containing protein OS=Fusi	1615	Fusicatenibacter sacchari	1	1	442	1	400	3817
Iron complex outermembrane recepter protei	689	Bacteroides uniformis	1	1	425	1	7	3501
phosphopyruvate hydratase OS=Gallintestini	429	Gallintestinimicrobium pr	1	60	219	216	1	3171
Oligo-1,6-glucosidase OS=Fusicatenibacter s	514	Fusicatenibacter sacchari	1	1	1	49	1	3157
beta-glucosidase BglX OS=Fusicatenibacter s	739	Fusicatenibacter sacchari	1	5	31	1	13	2974
Beta-galactosidase OS=Blautia_A fusiformis	722	Blautia_A fusiformis	1	40	1	31	4	2955
DUF4886 domain-containing protein OS=Alist	1413	Alistipes finegoldii	1	1	226	1	116	2928
ABC transporter, solute-binding protein OS=A	535	Anthropogastromicrobiun	1	45	1	1	28	2907
Alpha-xylosidase OS=Fusicatenibacter sacch	795	Fusicatenibacter sacchari	1	21	35	43	20	2808
Type VI secretion system needle protein Hcp C	132	Bacteroides uniformis	1	7	1	18	20	2674
DNA-directed RNA polymerase subunit beta O	1270	Parabacteroides merdae	1	251	102	556	234	2638
putative dehydrogenases and related protein	486	Alistipes onderdonkii	1	76	126	84	118	2579
Efflux RND transporter periplasmic adaptor s	484	Fusicatenibacter sacchari	1	60	39	1	1	2407
3-keto-disaccharide hydrolase domain-conta	1150	Parabacteroides merdae	1	194	125	38	402	2364

Table 2: Resistant dextrose effect as a prebiotic
Protein regulation in feces sample 24h after with different probiotics. Results are sorted by decreasing order of Resistant Dextrose, highlighting specific effect on some *Fusicatenibacter saccharivorans* enzymes.

Entry Name	Protein	Organism	Contol T0 S1-0h-NSC	Control S41-24h-NSC	Inulin S42-24h-IN	Pectin S43-24h-P	2-FL S44-24h-2FL	Resistant Dextrose S45-24h-RD
Multidomain with s-layer-like proteiny regior	1200	CAG-274 sp000432155	1	151	106	7370	137	84
Carbohydrate kinase PfkB domain-containing	349	CAG-274 sp000432155	1	193	217	5663	81	68
type I glutamate--ammonia ligase OS=CAG-27	443	CAG-274 sp000432155	1	12	1	5298	24	1
elongation factor G OS=CAG-274 sp00043215	705	CAG-274 sp000432155	1	59	43	3600	49	54
beta-ketoacyl-ACP synthase II OS=Faecalibact	412	Faecalibacterium prausni	1	90	1144	3414	1204	87
50S ribosomal protein L7/L12 OS=CAG-274 sp	125	CAG-274 sp000432155	1	118	105	3141	80	101
glucuronate isomerase OS=CAG-274 sp00043	467	CAG-274 sp000432155	1	23	25	2877	1	1
bifunctional 4-hydroxy-3-methylbut-2-enyl di	646	CAG-274 sp000432155	1	74	232	2656	335	1
hydroxylamine reductase OS=Megamonas fur	544	Megamonas funiformis	1	130	454	2650	375	17
pyruvate kinase OS=CAG-274 sp000432155 G	588	CAG-274 sp000432155	1	106	8	2546	181	36
30S ribosomal protein S7 OS=CAG-274 sp000	156	CAG-274 sp000432155	1	103	64	2491	65	62
30S ribosomal protein S5 OS=CAG-274 sp000	168	CAG-274 sp000432155	1	62	19	2466	52	47
bifunctional 4-hydroxy-2-oxoglutarate aldola	322	Megamonas funiformis	1	99	198	2429	309	219
30S ribosomal protein S4 OS=CAG-274 sp000	196	CAG-274 sp000432155	1	61	162	2333	45	301
50S ribosomal protein L2 OS=CAG-274 sp000	276	CAG-274 sp000432155	1	45	83	2129	42	19
Dockerin domain-containing protein OS=CAG	363	CAG-274 sp000432155	1	98	8	2118	85	23
50S ribosomal protein L5 OS=CAG-274 sp000	179	CAG-274 sp000432155	1	332	135	2112	128	149
carbamoyl-phosphate synthase large subunit	1072	CAG-274 sp000432155	1	71	118	1988	103	36
Pectate lyase OS=Prevotella copri_B	605	Prevotella copri_B	1	1	1	1973	1	8
50S ribosomal protein L1 OS=CAG-274 sp000	231	CAG-274 sp000432155	1	41	311	1934	20	215
Histidine kinase OS=Bifidobacterium adolesc	508	Bifidobacterium adolescer	1	1	19	1901	82	50
argininosuccinate synthase OS=CAG-274 sp0	405	CAG-274 sp000432155	1	18	64	1889	47	45

Table 1: Pectine effect as a prebiotic
Protein regulation in feces sample 24h after with different probiotics. Results are sorted by decreasing order of pectine response, highlighting the effect on CAG-274

Conclusion

- A routine metaproteomics approach highlighted species-specific activation of metabolic pathways
- We detected ecosystem and community-levels functional insights, beyond taxonomy
- We established mechanistic links to metabolites such as indole-3-lactic acid
- We obtained a functional validation of prebiotic and probiotic effects

Analytical flow Metaproteomics