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SCFA Analysis: Abbott Carbohydrate Obesity Project

Code ▾

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Introduction

Project Purpose and Significance

Obesity continues to increase as a public health emergency, with the origins of most adult obesity being in childhood. Effective clinical approaches are urgently needed to prevent or reverse childhood obesity. The gut-associated microbiome is an established central factor in energy harvest, hepatic function, insulin sensitivity, and adipose tissue homeostasis, making it a critical target for obesity intervention strategies.

This project represents a collaborative effort between Lurie Children's Hospital and Abbott Nutrition to investigate interpersonal variation in energetics and short-chain fatty acid (SCFA) production of obesity-associated gut microbiota in response to slow and fast digestible carbohydrates. The work builds upon

foundational research demonstrating that carbohydrate quality, rather than quantity alone, plays a crucial role in metabolic health.

Scientific Foundation and Motivation

The project was motivated by compelling evidence from the *Frontiers in Nutrition* paper by Wang et al. (2022) that demonstrated the therapeutic potential of slowly digestible carbohydrates (SDC) in metabolic syndrome and obesity management. This seminal work showed that SDC displays beneficial effects on reducing glucose excursions in healthy, insulin-resistant, and type 2 diabetic individuals, inducing a slow and prolonged glucose release that results in reduced postprandial glycemic responses and extended glycemic index values.

In type 2 diabetic patients, SDC-rich diets (60g/day) reduced glycemic variability parameters by 17-23%, with these parameters correlating with HbA1c, suggesting potential for long-term glycemic improvement. The *Frontiers* paper also demonstrated that foods with the highest SDC content (23.9-27.5 g/100g) induce the lowest glycemic responses with the lowest incremental AUC of glucose and insulin concentration.

Comparative Evidence from Murine Models

In comparison to fast digestible carbohydrate (FDC) sources, the Abbott group has shown in murine models of obesity that nutrition with slow digestible carbohydrates (SDC) reverses obesity-associated phenotypes, including elevated body mass, insulin resistance, and systemic inflammation. However, the interpersonal differences in SDC responses by the childhood-associated human microbiota may not be fully predicted in murine obesity models.

Interpersonal Variation in Human Microbiota

Lurie investigators and colleagues have shown interpersonal variation in the production of short-chain fatty acids (SCFA) by the human gut microbiome from adolescents with obesity in response to ex vivo prebiotic exposure, suggesting that complex carbohydrate utilization by the microbiota varies between individuals and thus may affect who responds to SDC and other nutritional approaches to obesity.

Understanding the variation in the compositional and metabolic responses of the childhood-associated microbiota may inform future obesity-treatment trials and precision approaches to obesity therapy.

Project Objectives

The main objectives of this project are to:

1. **Measure variation in responses** between different human gut-associated microbiome communities to FDC and SDC
2. **Identify childhood-associated organisms** with facile utilization of SDC
3. **Test interindividual variation** of short-chain fatty acid production among fecal microbiota samples to slow and fast digestible carbohydrates
4. **Measure energy harvest differences** between human obesity-associated fecal microbiota
5. **Isolate SDC bacterial utilizers** using single cell isolation techniques towards the future goal of creating obesity treatment symbiotic combinations

Clinical Impact

Understanding the interpersonal differences in SDC utilization by the childhood-associated human microbiota may inform future obesity-treatment trials through the identification of likely responders and, subsequently, precision approaches to obesity therapy. This precision nutrition approach could revolutionize childhood

obesity treatment by enabling personalized dietary interventions based on individual microbiome composition and metabolic capacity.

Analysis Overview

This document presents the analysis of short-chain fatty acids (SCFAs) in the Abbott carbohydrate obesity project. The analysis examines how SCFA analytes change between experimental groups, carbohydrate types, and time points, with particular focus on identifying interpersonal variation in metabolic responses to different carbohydrate sources.

Methods

Metabolomics Overview

The fecal metabolome was analyzed using targeted metabolomics approaches. The DFI Host-Microbe Metabolomics Facility (DFI-HMMF) analyzed fecal material using validated methods and analysis pipelines. All compounds were validated through retention time and fragmentation comparison to standards and available databases.

SCFA Analysis using PFBr Panel

Short chain fatty acids were analyzed using Gas chromatography-mass spectrometry (GC-MS) following derivatization with pentafluorobenzyl bromide (PFBr). SCFAs (acetate, butyrate, propionate) were quantitatively analyzed following PFB derivatization and detection by negative collision induced gas chromatography-mass spectrometry (($\text{--}\text{Cl}$)-GC-MS, Agilent 8890). Additional compounds including 5-aminovalerate and succinate were also quantified.

Detailed SCFA Analysis Protocol

The following section outlines the specific protocol used for SCFA derivatization and GC-MS analysis.

Short chain fatty acids were derivatized as described by Haak et al. with modifications. The metabolite extract (100 μL) was added to 100 μL of 100 mM borate buffer (pH 10), 400 μL of 100 mM pentafluorobenzyl bromide in Acetonitrile, and 400 μL of n-hexane in a capped mass spec autosampler vial. Samples were heated to 65°C for 1 hour while shaking at 1300 rpm. After cooling, samples were centrifuged at 4°C, 2000 $\times g$ for 5 min, allowing phase separation. The hexanes phase was transferred and analyzed.

Samples were analyzed using a GC-MS (Agilent 7890A GC system, Agilent 5975C MS detector) operating in negative chemical ionization mode, using a HP-5MSUI column (30 m \times 0.25 mm, 0.25 μm), methane as the reagent gas and 1 μL split injection (1:10 split ratio). A 10-point calibration curve was prepared with acetate (100 mM), propionate (25 mM), butyrate (12.5 mM), and succinate (50 mM), with 9 subsequent 2x serial dilutions.

Sample Extraction

This section describes the procedure for extracting metabolites from the fecal samples prior to analysis.

Extraction solvent (80% methanol spiked with internal standards and stored at -80°C) was added at a ratio of 100 mg of material/mL of extraction solvent. Samples were homogenized at 4°C on a Bead Mill 24 Homogenizer, set at 1.6 m/s with 6 thirty-second cycles, 5 seconds off per cycle. Samples were then centrifuged at -10°C, 20,000 $\times g$ for 15 min and the supernatant was used for analysis.

Data Analysis

Load Metadata

Load SCFA Data

```
## Dataset dimensions: 440 16
```

```
## Sample groups: Case Control
```

```
## Carbohydrate types: Rapid Digestible Slow Digestible No Carbohydrate
```

```
## Time points: 0 48
```

Summary Statistics

```
## Total observations after averaging technical replicates: 480
```

Summary by Group

Summary Statistics by Experimental Group

Group	Analyte	n	Mean	Median	SD	SEM	Q25	Q75
Case	5aminovalerate	48	0.485	0.245	0.561	0.081	0.050	0.701
Control	5aminovalerate	48	0.554	0.495	0.504	0.073	0.050	0.959
Case	acetate	48	17.304	13.109	16.795	2.424	1.201	32.258
Control	acetate	48	18.734	22.513	16.704	2.411	1.214	33.208
Case	butyrate	48	3.569	1.635	3.786	0.546	0.260	7.062
Control	butyrate	48	4.245	3.745	4.214	0.608	0.291	7.284
Case	propionate	48	2.733	1.393	2.955	0.427	0.170	4.450
Control	propionate	48	2.750	2.963	2.614	0.377	0.184	4.782
Case	succinate	48	0.779	0.170	1.244	0.180	0.080	1.238
Control	succinate	48	1.243	0.345	1.722	0.249	0.132	1.788

Summary by Carbohydrate Type

Summary Statistics by Carbohydrate Type

Carbohydrate Type	Analyte	n	Mean	Median	SD	SEM	Q25	Q75
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Carbohydrate Type	Analyte	n	Mean	Median	SD	SEM	Q25	Q75
No Carbohydrate	5aminovalerate	32	0.560	0.488	0.554	0.098	0.050	0.895
Rapid Digestible	5aminovalerate	32	0.489	0.392	0.513	0.091	0.050	0.919
Slow Digestible	5aminovalerate	32	0.509	0.392	0.541	0.096	0.050	0.787
No Carbohydrate	acetate	32	16.048	17.312	14.812	2.618	1.250	30.038
Rapid Digestible	acetate	32	18.233	23.961	17.056	3.015	1.152	33.003
Slow Digestible	acetate	32	19.776	25.528	18.295	3.234	1.188	36.237
No Carbohydrate	butyrate	32	3.201	3.150	2.956	0.523	0.312	6.232
Rapid Digestible	butyrate	32	4.000	2.799	4.396	0.777	0.279	7.631
Slow Digestible	butyrate	32	4.519	4.089	4.476	0.791	0.282	7.884
No Carbohydrate	propionate	32	3.206	3.470	3.125	0.552	0.188	6.151
Rapid Digestible	propionate	32	2.244	2.411	2.244	0.397	0.168	3.516
Slow Digestible	propionate	32	2.775	2.407	2.885	0.510	0.189	4.630
No Carbohydrate	succinate	32	0.691	0.262	0.979	0.173	0.000	0.700
Rapid Digestible	succinate	32	1.067	0.255	1.570	0.278	0.115	1.341
Slow Digestible	succinate	32	1.275	0.245	1.846	0.326	0.114	1.974

Summary by Time Point

Summary Statistics by Time Point

Time (Hours)	Analyte	n	Mean	Median	SD	SEM	Q25	Q75
0	5aminovalerate	48	0.088	0.050	0.112	0.016	0.050	0.066
48	5aminovalerate	48	0.950	0.900	0.423	0.061	0.677	1.177
0	acetate	48	2.484	1.208	5.378	0.776	1.043	1.322
48	acetate	48	33.554	33.065	6.347	0.916	29.494	38.398
0	butyrate	48	0.546	0.272	0.949	0.137	0.230	0.413
48	butyrate	48	7.267	7.119	2.890	0.417	4.744	8.834
0	propionate	48	0.362	0.170	0.735	0.106	0.135	0.233
48	propionate	48	5.122	4.815	1.860	0.268	3.550	6.586
0	succinate	48	0.280	0.125	0.664	0.096	0.076	0.236
48	succinate	48	1.742	1.327	1.758	0.254	0.404	2.714

Combined Summary Statistics

```
## Combined summary table contains 60 condition combinations
```

```
## Combined summary saved to results/combined_summary_statistics.csv
```

Statistical Analysis

```
## Subject-level and case-only analyses saved to results/ directory
```

Statistical Results

We performed several statistical tests to compare SCFA concentrations across different experimental conditions. The following tables summarize the results of these comparisons, including t-tests for group differences and ANOVA for carbohydrate effects.

Group Comparisons (Control vs Case)

This table presents the results of t-tests comparing SCFA concentrations between the control and case groups.

Group Comparisons with Benjamini-Hochberg Correction

Analyte	.y.	group1	group2	n1	n2	statistic	df	P-value	Adjusted	
									P-value	Significance
5aminovalerate	concentration	control	case	48	48	0.6290	92.9323	0.531	0.8463	ns
acetate	concentration	control	case	48	48	0.4183	93.9972	0.677	0.8463	ns
butyrate	concentration	control	case	48	48	0.8265	92.9395	0.411	0.8463	ns
propionate	concentration	control	case	48	48	0.0300	92.6226	0.976	0.9760	ns
succinate	concentration	control	case	48	48	1.5115	85.5443	0.134	0.6700	ns

Carbohydrate Type Comparisons

This table shows the results of ANOVA tests examining the effect of different carbohydrate types on SCFA concentrations.

ANOVA Results for Carbohydrate Type Effects

Analyte	Effect	DFn	DFd	F	P-value	p<.05	Adjusted P-		
							ges	value	Significance
5aminovalerate	carbohydrate_type	2	93	0.147	0.864		0.003		0.8640 ns
acetate	carbohydrate_type	2	93	0.399	0.672		0.008		0.8400 ns
butyrate	carbohydrate_type	2	93	0.880	0.418		0.019		0.6967 ns
propionate	carbohydrate_type	2	93	0.965	0.385		0.020		0.6967 ns

Analyte	Effect	DFn	DFd	F	P-value	p<.05	Adjusted P-	
							P-value	Significance
succinate	carbohydrate_type	2	93	1.233	0.296	0.026	0.6967	ns

Post-hoc Carbohydrate Comparisons

Following the ANOVA, pairwise t-tests were performed to compare each carbohydrate type to the 'no carbohydrate' control. The results are shown below.

Pairwise Comparisons vs No Carbohydrate Control

Analyte	.y.	group1	group2	n1	n2	P-value	Adjusted	
							p.signif	P-value
Saminovalerate	concentration	no_carbohydrate	rapid_digestible	32	32	0.601	ns	0.6711 ns
Saminovalerate	concentration	no_carbohydrate	slow_digestible	32	32	0.706	ns	0.7060 ns
acetate	concentration	no_carbohydrate	rapid_digestible	32	32	0.604	ns	0.6711 ns
acetate	concentration	no_carbohydrate	slow_digestible	32	32	0.377	ns	0.6711 ns
butyrate	concentration	no_carbohydrate	rapid_digestible	32	32	0.427	ns	0.6711 ns
butyrate	concentration	no_carbohydrate	slow_digestible	32	32	0.191	ns	0.6367 ns
propionate	concentration	no_carbohydrate	rapid_digestible	32	32	0.169	ns	0.6367 ns
propionate	concentration	no_carbohydrate	slow_digestible	32	32	0.535	ns	0.6711 ns
succinate	concentration	no_carbohydrate	rapid_digestible	32	32	0.322	ns	0.6711 ns
succinate	concentration	no_carbohydrate	slow_digestible	32	32	0.125	ns	0.6367 ns

Three-way Interaction Analysis

To assess the combined effects of group, carbohydrate type, and time, a three-way ANOVA was conducted. The results are summarized in this table.

Three-way ANOVA: Group × Carbohydrate × Time Interactions

Analyte	Effect	P-value	Adjusted P-value	Significance
Saminovalerate	group	0.300	0.6125	ns
Saminovalerate	carbohydrate_type	0.666	0.9212	ns
Saminovalerate	timepoint_hr	0.000	0.0000	****
Saminovalerate	group:carbohydrate_type	0.935	0.9680	ns
Saminovalerate	group:timepoint_hr	0.706	0.9212	ns
Saminovalerate	carbohydrate_type:timepoint_hr	0.731	0.9212	ns
Saminovalerate	group:carbohydrate_type:timepoint_hr	0.942	0.9680	ns
acetate	group	0.222	0.5180	ns

Analyte	Effect	P-value	Adjusted P-value	Significance
acetate	carbohydrate_type	0.036	0.1435	ns
acetate	timepoint_hr	0.000	0.0000	****
acetate	group:carbohydrate_type	0.440	0.8105	ns
acetate	group:timepoint_hr	0.315	0.6125	ns
acetate	carbohydrate_type:timepoint_hr	0.114	0.3069	ns
acetate	group:carbohydrate_type:timepoint_hr	0.737	0.9212	ns
butyrate	group	0.112	0.3069	ns
butyrate	carbohydrate_type	0.041	0.1435	ns
butyrate	timepoint_hr	0.000	0.0000	****
butyrate	group:carbohydrate_type	0.576	0.9164	ns
butyrate	group:timepoint_hr	0.658	0.9212	ns
butyrate	carbohydrate_type:timepoint_hr	0.038	0.1435	ns
butyrate	group:carbohydrate_type:timepoint_hr	0.571	0.9164	ns
propionate	group	0.951	0.9680	ns
propionate	carbohydrate_type	0.022	0.1283	ns
propionate	timepoint_hr	0.000	0.0000	****
propionate	group:carbohydrate_type	0.963	0.9680	ns
propionate	group:timepoint_hr	0.256	0.5600	ns
propionate	carbohydrate_type:timepoint_hr	0.027	0.1350	ns
propionate	group:carbohydrate_type:timepoint_hr	0.950	0.9680	ns
succinate	group	0.095	0.3023	ns
succinate	carbohydrate_type	0.218	0.5180	ns
succinate	timepoint_hr	0.000	0.0000	****
succinate	group:carbohydrate_type	0.960	0.9680	ns
succinate	group:timepoint_hr	0.659	0.9212	ns
succinate	carbohydrate_type:timepoint_hr	0.492	0.8610	ns
succinate	group:carbohydrate_type:timepoint_hr	0.968	0.9680	ns

Subject-Level Analyses

To account for individual variability, we conducted analyses at the subject level. This allows us to examine within-subject changes and summarize statistics for each participant, providing a more granular view of the data.

Subject-Level Summary Statistics

This table provides summary statistics for each subject, including the mean concentration and number of observations.

Subject-Level Summary Statistics

Group	Analyte	n Subjects	Mean Subject Means	SD Subject Means	SEM Subjects
control	5aminovalerate	8	0.554	0.124	0.044
control	acetate	8	18.734	5.296	1.872
control	butyrate	8	4.245	1.321	0.467
control	propionate	8	2.750	0.897	0.317
control	succinate	8	1.243	1.137	0.402
case	5aminovalerate	8	0.485	0.276	0.098
case	acetate	8	17.304	2.657	0.940
case	butyrate	8	3.569	1.103	0.390
case	propionate	8	2.733	0.999	0.353
case	succinate	8	0.779	0.707	0.250

Within-Subject Changes (0h to 48h)

This table shows the results of statistical tests on the changes in SCFA concentrations within each subject from baseline to 48 hours.

Within-Subject Changes from Baseline to 48h

Group	Carbohydrate		n	Mean	SD	SEM	t-	P-value	P-value	Adjusted Significance
	Type	Analyte		Change	Change	Change	statistic			
control	no_carbohydrate	5aminovalerate	8	0.9675	0.4641	0.1641	5.8963	0.0006	0.0009	***
control	no_carbohydrate	acetate	8	26.0637	8.4143	2.9749	8.7612	0.0001	0.0001	***
control	no_carbohydrate	butyrate	8	4.8569	1.9468	0.6883	7.0564	0.0002	0.0003	***
control	no_carbohydrate	propionate	8	5.2625	1.4668	0.5186	10.1473	0.0000	0.0001	***
control	no_carbohydrate	succinate	8	1.2794	1.1714	0.4141	3.0892	0.0176	0.0657	ns
control	rapid_digestible	5aminovalerate	8	0.7974	0.2539	0.0898	8.8830	0.0000	0.0001	***
control	rapid_digestible	acetate	8	31.2600	6.8995	2.4393	12.8150	0.0000	0.0000	***
control	rapid_digestible	butyrate	8	7.3123	4.1611	1.4712	4.9704	0.0016	0.0017	**
control	rapid_digestible	propionate	8	3.4114	1.8180	0.6428	5.3073	0.0011	0.0011	**
control	rapid_digestible	succinate	8	1.5103	2.0037	0.7084	2.1320	0.0705	0.0705	ns
control	slow_digestible	5aminovalerate	8	0.8964	0.2789	0.0986	9.0918	0.0000	0.0001	***

Group	Type	Analyte	Mean		SD Change	SEM Change	t-statistic	P-value	Adjusted P-value		Significance
			n	Change					P-value		
control	slow_digestible	acetate	8	32.3582	8.0537	2.8474	11.3640	0.0000	0.0000	***	
control	slow_digestible	butyrate	8	8.5557	3.3129	1.1713	7.3045	0.0002	0.0003	***	
control	slow_digestible	propionate	8	4.6503	1.9143	0.6768	6.8711	0.0002	0.0004	***	
control	slow_digestible	succinate	8	1.9625	2.2616	0.7996	2.4544	0.0438	0.0657	ns	
case	no_carbohydrate	5aminovalerate	8	0.8937	0.3891	0.1376	6.4970	0.0003	0.0007	***	
case	no_carbohydrate	acetate	8	29.9688	3.4826	1.2313	24.3397	0.0000	0.0000	***	
case	no_carbohydrate	butyrate	8	5.7231	1.1415	0.4036	14.1811	0.0000	0.0000	***	
case	no_carbohydrate	propionate	8	6.0431	1.8893	0.6680	9.0472	0.0000	0.0001	***	
case	no_carbohydrate	succinate	8	0.8456	1.1009	0.3892	2.1726	0.0664	0.0705	ns	
case	rapid_digestible	5aminovalerate	8	0.8112	0.5696	0.2014	4.0281	0.0050	0.0057	**	
case	rapid_digestible	acetate	8	31.0581	6.9134	2.4442	12.7066	0.0000	0.0000	***	
case	rapid_digestible	butyrate	8	6.5300	3.7671	1.3319	4.9029	0.0017	0.0017	**	
case	rapid_digestible	propionate	8	4.1556	1.5547	0.5497	7.5602	0.0001	0.0003	***	
case	rapid_digestible	succinate	8	1.4047	1.5458	0.5465	2.5702	0.0370	0.0657	ns	
case	slow_digestible	5aminovalerate	8	0.8075	0.5815	0.2056	3.9275	0.0057	0.0057	**	
case	slow_digestible	acetate	8	35.7078	5.2959	1.8724	19.0709	0.0000	0.0000	***	
case	slow_digestible	butyrate	8	7.3503	2.2995	0.8130	9.0408	0.0000	0.0001	***	
case	slow_digestible	propionate	8	5.0372	2.3603	0.8345	6.0363	0.0005	0.0006	***	
case	slow_digestible	succinate	8	1.7725	1.7861	0.6315	2.8069	0.0263	0.0657	ns	

Case-Only Temporal Analysis

To isolate the effects of the intervention within the case group, we performed a temporal analysis comparing SCFA concentrations at 0h and 48h. This analysis helps to understand the direct impact of the carbohydrate types on the case subjects over time.

Case Group Temporal Changes by Carbohydrate Type

This table presents the temporal changes in SCFA concentrations for the case group, broken down by carbohydrate type.

Case Group Only: Temporal Changes (0h to 48h) by Carbohydrate Type

Carbohydrate Type	Analyte	Mean		SD Change	SEM Change	t-statistic	P-value	Adjusted P-value		Significance
		n	Change					P-value		
no_carbohydrate	5aminovalerate	8	0.8937	0.3891	0.1376	6.4970	0.0003	0.0010	**	
no_carbohydrate	acetate	8	29.9688	3.4826	1.2313	24.3397	0.0000	0.0000	***	

Carbohydrate			Mean	SD	SEM	t-statistic	P-value	Adjusted P-value	Adjusted Significance
Type	Analyte	n	Change	Change	Change				
no_carbohydrate	butyrate	8	5.7231	1.1415	0.4036	14.1811	0.0000	0.0000	***
no_carbohydrate	propionate	8	6.0431	1.8893	0.6680	9.0472	0.0000	0.0001	***
no_carbohydrate	succinate	8	0.8456	1.1009	0.3892	2.1726	0.0664	0.0664	ns
rapid_digestible	5aminovalerate	8	0.8112	0.5696	0.2014	4.0281	0.0050	0.0057	**
rapid_digestible	acetate	8	31.0581	6.9134	2.4442	12.7066	0.0000	0.0000	***
rapid_digestible	butyrate	8	6.5300	3.7671	1.3319	4.9029	0.0017	0.0017	**
rapid_digestible	propionate	8	4.1556	1.5547	0.5497	7.5602	0.0001	0.0002	***
rapid_digestible	succinate	8	1.4047	1.5458	0.5465	2.5702	0.0370	0.0555	ns
slow_digestible	5aminovalerate	8	0.8075	0.5815	0.2056	3.9275	0.0057	0.0057	**
slow_digestible	acetate	8	35.7078	5.2959	1.8724	19.0709	0.0000	0.0000	***
slow_digestible	butyrate	8	7.3503	2.2995	0.8130	9.0408	0.0000	0.0001	***
slow_digestible	propionate	8	5.0372	2.3603	0.8345	6.0363	0.0005	0.0005	***
slow_digestible	succinate	8	1.7725	1.7861	0.6315	2.8069	0.0263	0.0555	ns

Case Group Temporal Changes (Pooled)

Here, we present the temporal changes for the case group, pooled across all carbohydrate types to assess the overall time effect.

Case Group Only: Temporal Changes (0h to 48h) Pooled Across Carbohydrate Types

Analyte	n	Mean Change	SD Change	SEM Change	t-statistic	P-value	Adjusted P-value	Significance
5aminovalerate	24	0.8375	0.4994	0.1019	8.2155	0e+00	0e+00	***
acetate	24	32.2449	5.7651	1.1768	27.4006	0e+00	0e+00	***
butyrate	24	6.5345	2.6049	0.5317	12.2894	0e+00	0e+00	***
propionate	24	5.0786	2.0342	0.4152	12.2310	0e+00	0e+00	***
succinate	24	1.3409	1.4895	0.3040	4.4104	2e-04	2e-04	***

Case Group Mixed-Effects Models (Subject Random Effects)

This table summarizes the results from the mixed-effects models applied to the case group data, accounting for subject-specific random effects.

Case Group Mixed-Effects Models: Time × Carbohydrate Effects with Subject Random Effects

Analyte	Effect	F-value	P-value	Significance
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Analyte	Effect	F-value	P-value	Significance
acetate	timepoint_hr	1481.9514	0.0000	***
acetate	carbohydrate_type	4.3256	0.0199	•
acetate	timepoint_hr:carbohydrate_type	4.4140	0.0185	•
butyrate	timepoint_hr	206.1035	0.0000	***
butyrate	carbohydrate_type	1.0672	0.3536	ns
butyrate	timepoint_hr:carbohydrate_type	1.0650	0.3543	ns
propionate	timepoint_hr	281.7401	0.0000	***
propionate	carbohydrate_type	3.6395	0.0353	•
propionate	timepoint_hr:carbohydrate_type	3.2477	0.0493	•
5aminovalerate	timepoint_hr	107.8313	0.0000	***
5aminovalerate	carbohydrate_type	0.1321	0.8766	ns
5aminovalerate	timepoint_hr:carbohydrate_type	0.1218	0.8857	ns
succinate	timepoint_hr	32.6569	0.0000	***
succinate	carbohydrate_type	1.5134	0.2325	ns
succinate	timepoint_hr:carbohydrate_type	1.3187	0.2789	ns

Delta Change Analysis (48h vs 0h)

To further investigate the magnitude of change over time, we calculated the delta (change) in concentration for each analyte between 48h and 0h. This approach focuses on the *response magnitude* and allows us to test whether the group or carbohydrate type influences how strongly subjects respond to the intervention over time.

Delta Summary Statistics

This table provides summary statistics for the calculated delta values (48h - 0h), showing the mean change and variability.

Summary Statistics for Delta Change (48h - 0h)

Group	Carbohydrate Type	Analyte	n	Mean Delta	SD Delta	SEM Delta
control	no_carbohydrate	5aminovalerate	8	0.967	0.464	0.164
control	no_carbohydrate	acetate	8	26.064	8.414	2.975
control	no_carbohydrate	butyrate	8	4.857	1.947	0.688
control	no_carbohydrate	propionate	8	5.263	1.467	0.519
control	no_carbohydrate	succinate	8	1.279	1.171	0.414

Group	Carbohydrate Type	Analyte	n	Mean	Delta	SD	Delta	SEM	Delta
control	rapid_digestible	5aminovalerate	8	0.797	0.254	0.090			
control	rapid_digestible	acetate	8	31.260	6.899	2.439			
control	rapid_digestible	butyrate	8	7.312	4.161	1.471			
control	rapid_digestible	propionate	8	3.411	1.818	0.643			
control	rapid_digestible	succinate	8	1.510	2.004	0.708			
control	slow_digestible	5aminovalerate	8	0.896	0.279	0.099			
control	slow_digestible	acetate	8	32.358	8.054	2.847			
control	slow_digestible	butyrate	8	8.556	3.313	1.171			
control	slow_digestible	propionate	8	4.650	1.914	0.677			
control	slow_digestible	succinate	8	1.962	2.262	0.800			
case	no_carbohydrate	5aminovalerate	8	0.894	0.389	0.138			
case	no_carbohydrate	acetate	8	29.969	3.483	1.231			
case	no_carbohydrate	butyrate	8	5.723	1.141	0.404			
case	no_carbohydrate	propionate	8	6.043	1.889	0.668			
case	no_carbohydrate	succinate	8	0.846	1.101	0.389			
case	rapid_digestible	5aminovalerate	8	0.811	0.570	0.201			
case	rapid_digestible	acetate	8	31.058	6.913	2.444			
case	rapid_digestible	butyrate	8	6.530	3.767	1.332			
case	rapid_digestible	propionate	8	4.156	1.555	0.550			
case	rapid_digestible	succinate	8	1.405	1.546	0.547			
case	slow_digestible	5aminovalerate	8	0.807	0.582	0.206			
case	slow_digestible	acetate	8	35.708	5.296	1.872			
case	slow_digestible	butyrate	8	7.350	2.300	0.813			
case	slow_digestible	propionate	8	5.037	2.360	0.834			
case	slow_digestible	succinate	8	1.772	1.786	0.631			

Delta Group Comparisons

This table shows the results of t-tests comparing the delta values between the control and case groups.

Group Comparisons of Delta Change (Response Magnitude)

analyte	.y.	group1	group2	n1	n2	statistic	df	p	p.adj	p.adj.signif
5aminovalerate	delta_48h_0h	control	case	24	24	0.4030	40.3837	0.689	0.6890	ns
acetate	delta_48h_0h	control	case	24	24	-1.1701	41.8753	0.249	0.6525	ns

analyte	.y.	group1	group2	n1	n2	statistic	df	p	p.adj	p.adj.signif
butyrate	delta_48h_0h	control	case	24	24	0.4200	42.5161	0.677	0.6890	ns
propionate	delta_48h_0h	control	case	24	24	-1.1375	45.5565	0.261	0.6525	ns
succinate	delta_48h_0h	control	case	24	24	0.5079	44.3472	0.614	0.6890	ns

Delta Carbohydrate Comparisons

This table presents the results of ANOVA tests on the delta values to examine the effect of carbohydrate type on the magnitude of change.

ANOVA Results for Carbohydrate Effects on Delta Change

analyte	Effect	DFn	DFd	F	p	p<.05	ges	p.adj	p.adj.signif
5aminovalerate	carbohydrate_type	2	45	0.355	0.703		0.016	0.703	ns
acetate	carbohydrate_type	2	45	3.253	0.048	•	0.126	0.080	ns
butyrate	carbohydrate_type	2	45	3.404	0.042	•	0.131	0.080	ns
propionate	carbohydrate_type	2	45	4.218	0.021	•	0.158	0.080	ns
succinate	carbohydrate_type	2	45	0.956	0.392		0.041	0.490	ns

Delta Carbohydrate Post-Hoc Comparisons

Following the ANOVA on delta values, pairwise t-tests were performed to compare each carbohydrate type to the 'no carbohydrate' control. The results are shown below.

Pairwise Comparisons of Delta Change vs No Carbohydrate

analyte	.y.	group1	group2	n1	n2	p	p.signif	p.adj	p.adj.signif
5aminovalerate	delta_48h_0h	no_carbohydrate	rapid_digestible	16	16	0.4090	ns	0.5112	ns
5aminovalerate	delta_48h_0h	no_carbohydrate	slow_digestible	16	16	0.6060	ns	0.6060	ns
acetate	delta_48h_0h	no_carbohydrate	rapid_digestible	16	16	0.1900	ns	0.3100	ns
acetate	delta_48h_0h	no_carbohydrate	slow_digestible	16	16	0.0143	•	0.0477	•
butyrate	delta_48h_0h	no_carbohydrate	rapid_digestible	16	16	0.1200	ns	0.3000	ns
butyrate	delta_48h_0h	no_carbohydrate	slow_digestible	16	16	0.0130	•	0.0477	•
propionate	delta_48h_0h	no_carbohydrate	rapid_digestible	16	16	0.0058	**	0.0477	•
propionate	delta_48h_0h	no_carbohydrate	slow_digestible	16	16	0.2170	ns	0.3100	ns
succinate	delta_48h_0h	no_carbohydrate	rapid_digestible	16	16	0.5010	ns	0.5567	ns
succinate	delta_48h_0h	no_carbohydrate	slow_digestible	16	16	0.1740	ns	0.3100	ns

Delta Interaction Analysis

To assess the combined effects of group and carbohydrate type on the delta values, a two-way ANOVA was conducted. The results are summarized in this table.

Two-way ANOVA on Delta Change: Group × Carbohydrate Interactions

Analyte	Effect	P-value	Adjusted P-value	Significance
Saminovalerate	group	0.699	0.8975	ns
Saminovalerate	carbohydrate_type	0.718	0.8975	ns
Saminovalerate	group:carbohydrate_type	0.939	0.9610	ns
acetate	group	0.233	0.7230	ns
acetate	carbohydrate_type	0.051	0.2550	ns
acetate	group:carbohydrate_type	0.647	0.8975	ns
butyrate	group	0.665	0.8975	ns
butyrate	carbohydrate_type	0.048	0.2550	ns
butyrate	group:carbohydrate_type	0.584	0.8975	ns
propionate	group	0.241	0.7230	ns
propionate	carbohydrate_type	0.024	0.2550	ns
propionate	group:carbohydrate_type	0.947	0.9610	ns
succinate	group	0.622	0.8975	ns
succinate	carbohydrate_type	0.414	0.8975	ns
succinate	group:carbohydrate_type	0.961	0.9610	ns

Delta Mixed-Effects Models

This table summarizes the results from the mixed-effects models applied to the delta values, accounting for subject-specific random effects.

Delta Change Mixed-Effects Models: Group × Carbohydrate Effects

Analyte	Effect	F-value	P-value	Significance
acetate	group	0.6260	0.4404	ns
acetate	carbohydrate_type	22.7732	0.0000	***
acetate	group:carbohydrate_type	3.1210	0.0578	ns
butyrate	group	0.0965	0.7601	ns
butyrate	carbohydrate_type	10.0422	0.0004	***
butyrate	group:carbohydrate_type	1.6684	0.2045	ns
propionate	group	0.6049	0.4481	ns

Analyte	Effect	F-value	P-value	Significance
propionate	carbohydrate_type	28.3790	0.0000	***
propionate	group:carbohydrate_type	0.3822	0.6854	ns
5aminovalerate	group	0.0709	0.7935	ns
5aminovalerate	carbohydrate_type	1.3520	0.2731	ns
5aminovalerate	group:carbohydrate_type	0.2555	0.7761	ns
succinate	group	0.1096	0.7449	ns
succinate	carbohydrate_type	4.7456	0.0157	•
succinate	group:carbohydrate_type	0.2126	0.8096	ns

Mixed-Effects Model Results

To account for the repeated measures design and the variability between subjects, we utilized linear mixed-effects models. These models include subject as a random effect, allowing us to more accurately assess the fixed effects of group, carbohydrate type, and time.

Mixed-Effects Model Summary

This table presents a summary of the full mixed-effects models, including F-values and p-values for all fixed effects.

Mixed-Effects Models: F-values and P-values for Fixed Effects

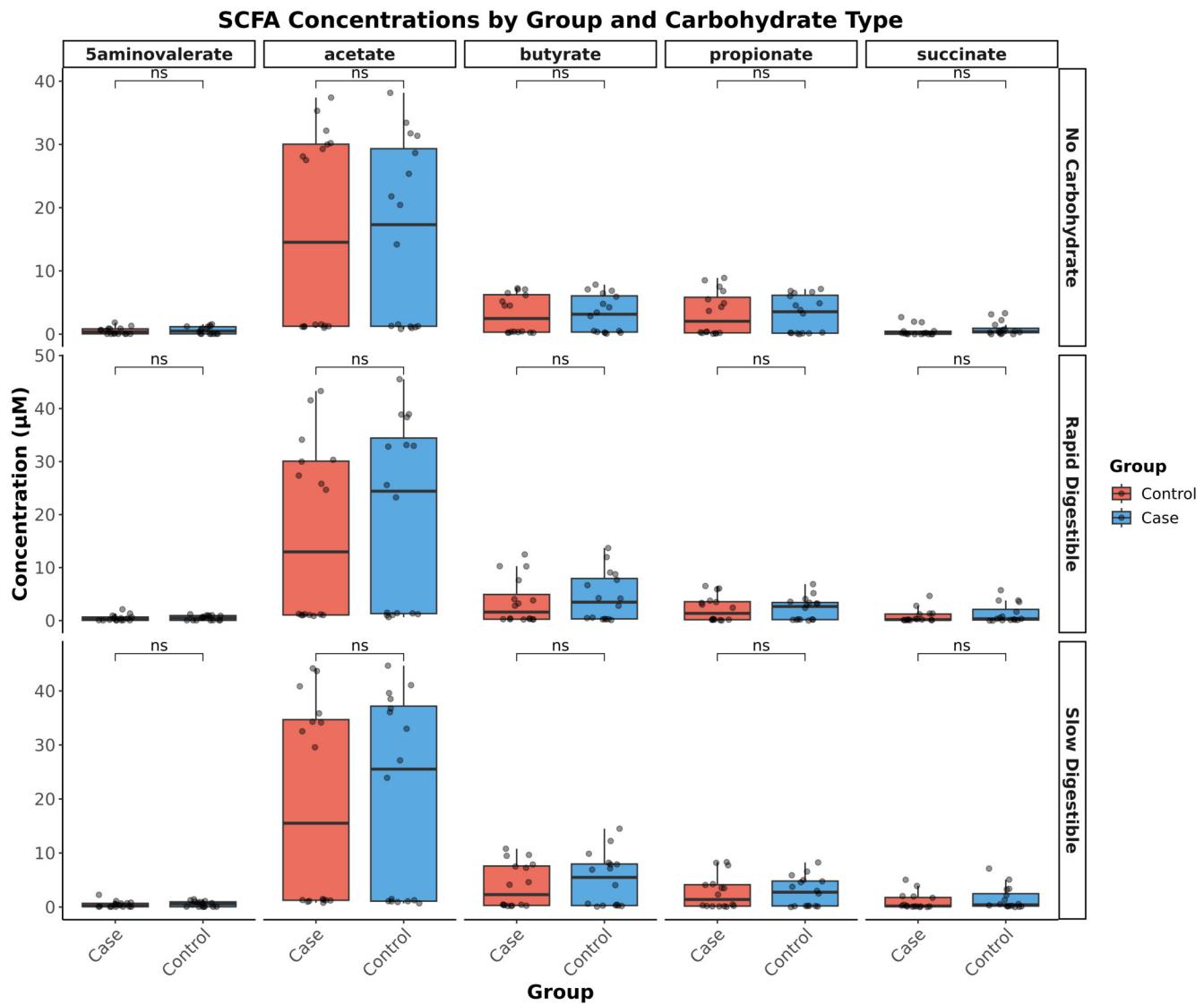
Analyte	Effect	F-value	P-value	Significance
acetate	group	0.5326	0.4761	ns
acetate	carbohydrate_type	7.1680	0.0014	**
acetate	timepoint_hr	1479.2346	0.0000	***
acetate	group:carbohydrate_type	1.7167	0.1862	ns
acetate	group:timepoint_hr	2.1173	0.1496	ns
acetate	carbohydrate_type:timepoint_hr	4.6260	0.0126	•
acetate	group:carbohydrate_type:timepoint_hr	0.6340	0.5331	ns
butyrate	group	1.4101	0.2524	ns
butyrate	carbohydrate_type	4.8623	0.0102	•
butyrate	timepoint_hr	373.5928	0.0000	***
butyrate	group:carbohydrate_type	0.8111	0.4480	ns
butyrate	group:timepoint_hr	0.2889	0.5924	ns
butyrate	carbohydrate_type:timepoint_hr	4.9696	0.0092	**

Analyte	Effect	F-value	P-value	Significance
butyrate	group:carbohydrate_type:timepoint_hr	0.8256	0.4417	ns
propionate	group	0.0015	0.9697	ns
propionate	carbohydrate_type	7.3837	0.0011	**
propionate	timepoint_hr	539.7055	0.0000	***
propionate	group:carbohydrate_type	0.0689	0.9335	ns
propionate	group:timepoint_hr	2.4183	0.1239	ns
propionate	carbohydrate_type:timepoint_hr	6.9780	0.0016	**
propionate	group:carbohydrate_type:timepoint_hr	0.0940	0.9104	ns
5aminovalerate	group	0.4662	0.5045	ns
5aminovalerate	carbohydrate_type	0.7003	0.4995	ns
5aminovalerate	timepoint_hr	296.3213	0.0000	***
5aminovalerate	group:carbohydrate_type	0.1157	0.8909	ns
5aminovalerate	group:timepoint_hr	0.2449	0.6220	ns
5aminovalerate	carbohydrate_type:timepoint_hr	0.5405	0.5846	ns
5aminovalerate	group:carbohydrate_type:timepoint_hr	0.1021	0.9030	ns
succinate	group	1.0964	0.3106	ns
succinate	carbohydrate_type	2.9249	0.0594	ns
succinate	timepoint_hr	53.4997	0.0000	***
succinate	group:carbohydrate_type	0.0777	0.9253	ns
succinate	group:timepoint_hr	0.3696	0.5449	ns
succinate	carbohydrate_type:timepoint_hr	1.3509	0.2649	ns
succinate	group:carbohydrate_type:timepoint_hr	0.0605	0.9413	ns

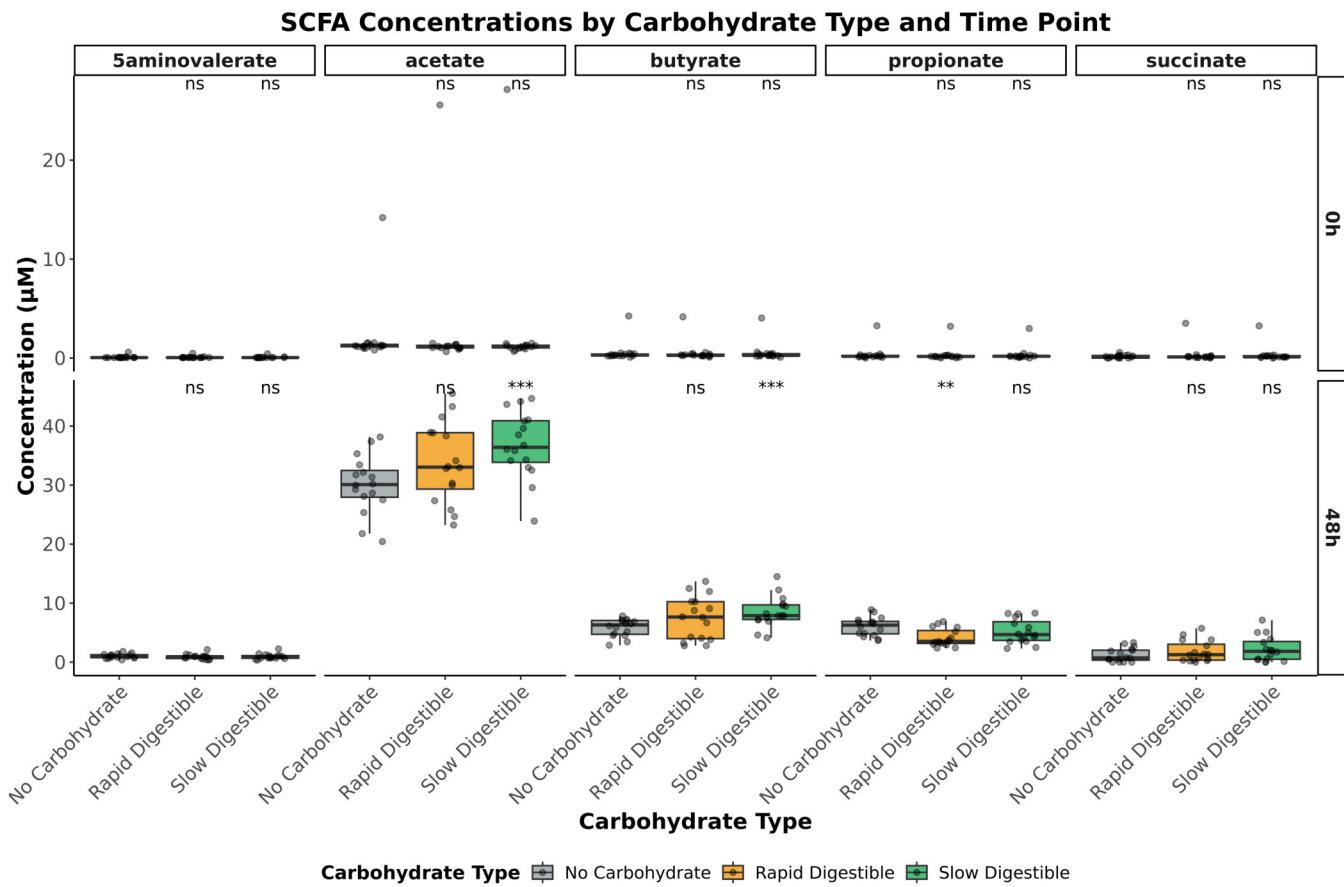
Visualizations

To visually explore the data, we generated a series of plots. These visualizations illustrate the relationships between SCFA concentrations and the experimental variables, including group, carbohydrate type, and time. Each plot is designed to highlight different aspects of the data, from overall trends to individual subject responses.

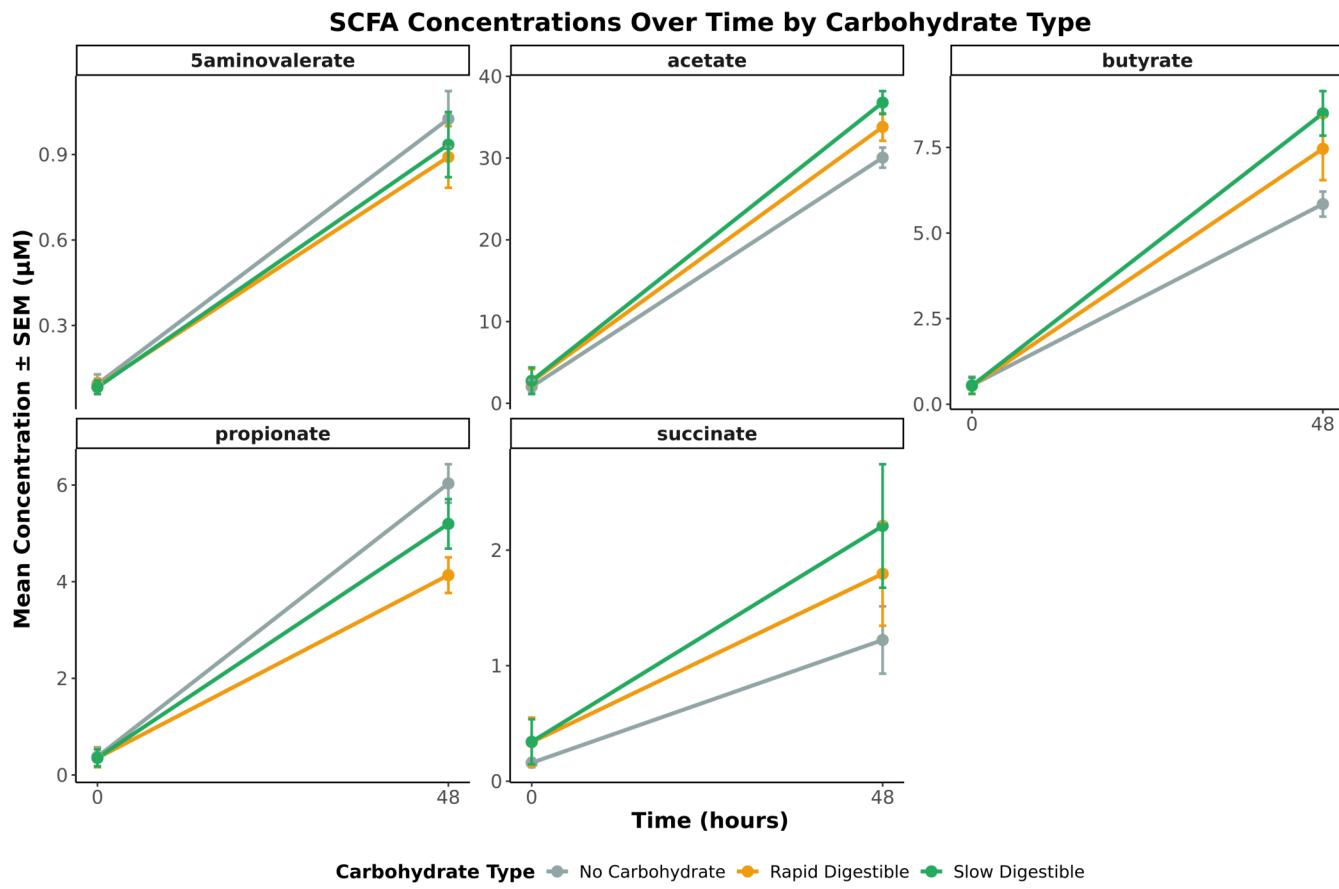
SCFA Concentrations by Group and Carbohydrate Type



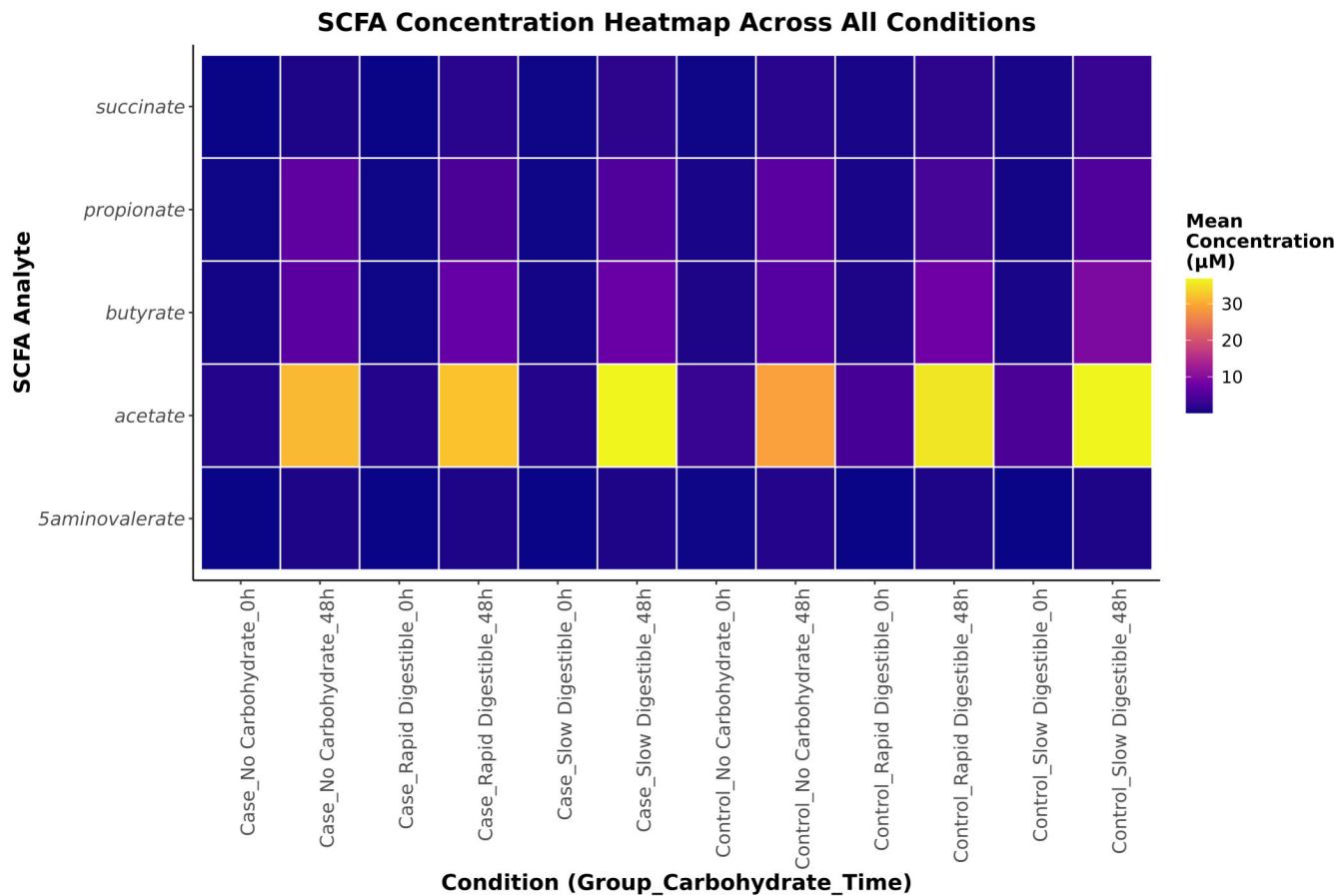
SCFA Concentrations by Carbohydrate Type and Time Point



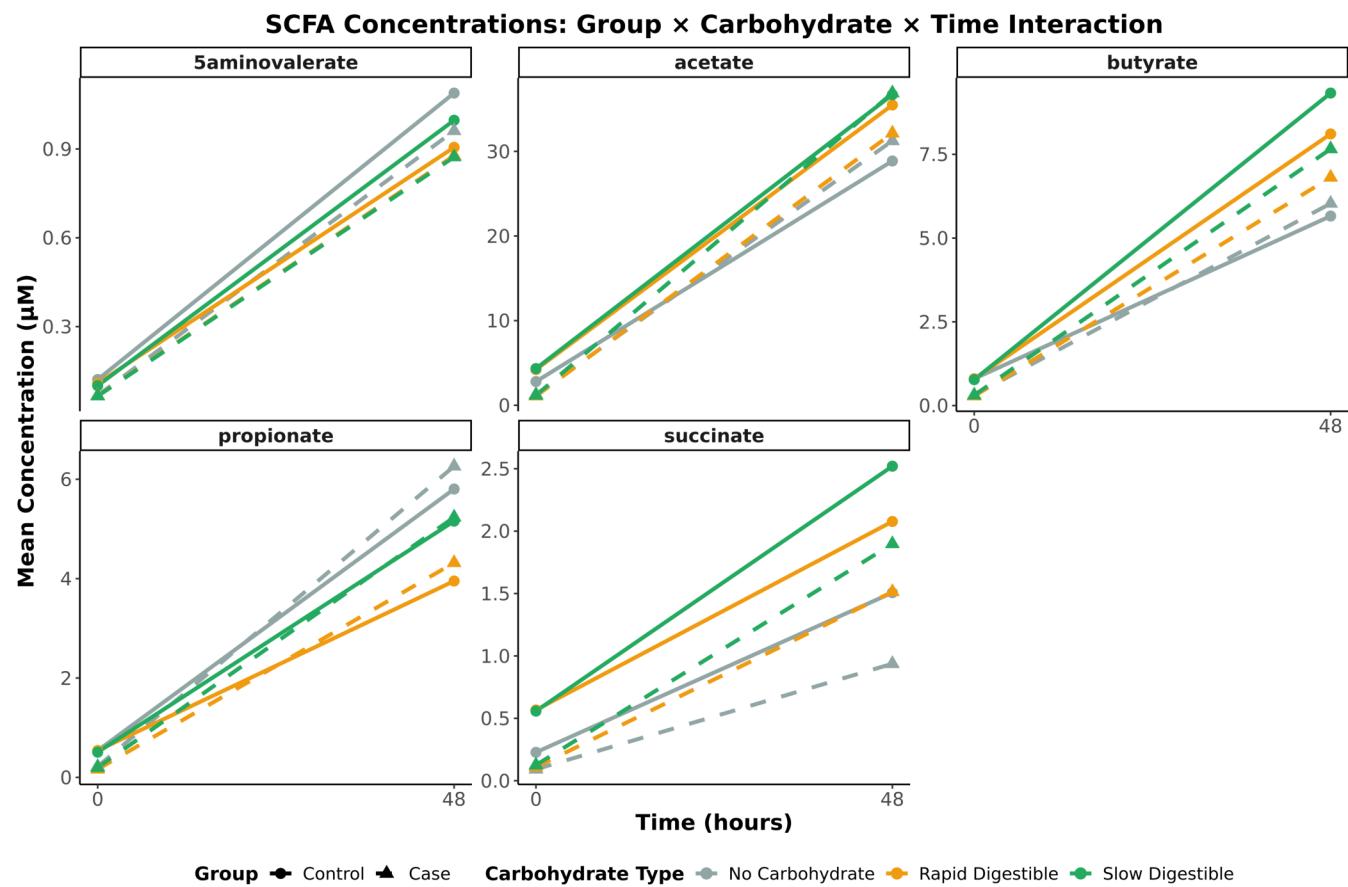
Time Series Analysis



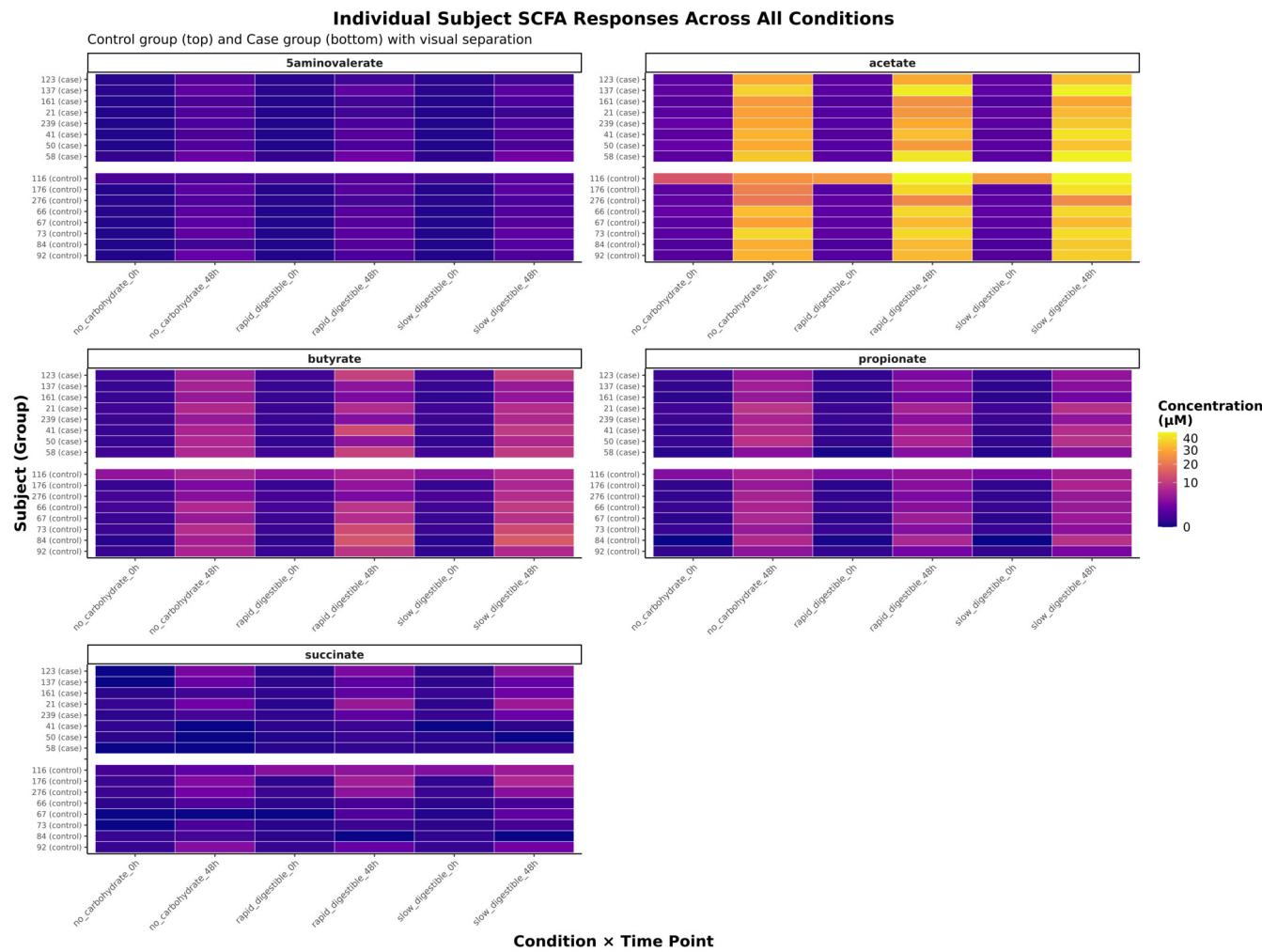
Concentration Heatmap



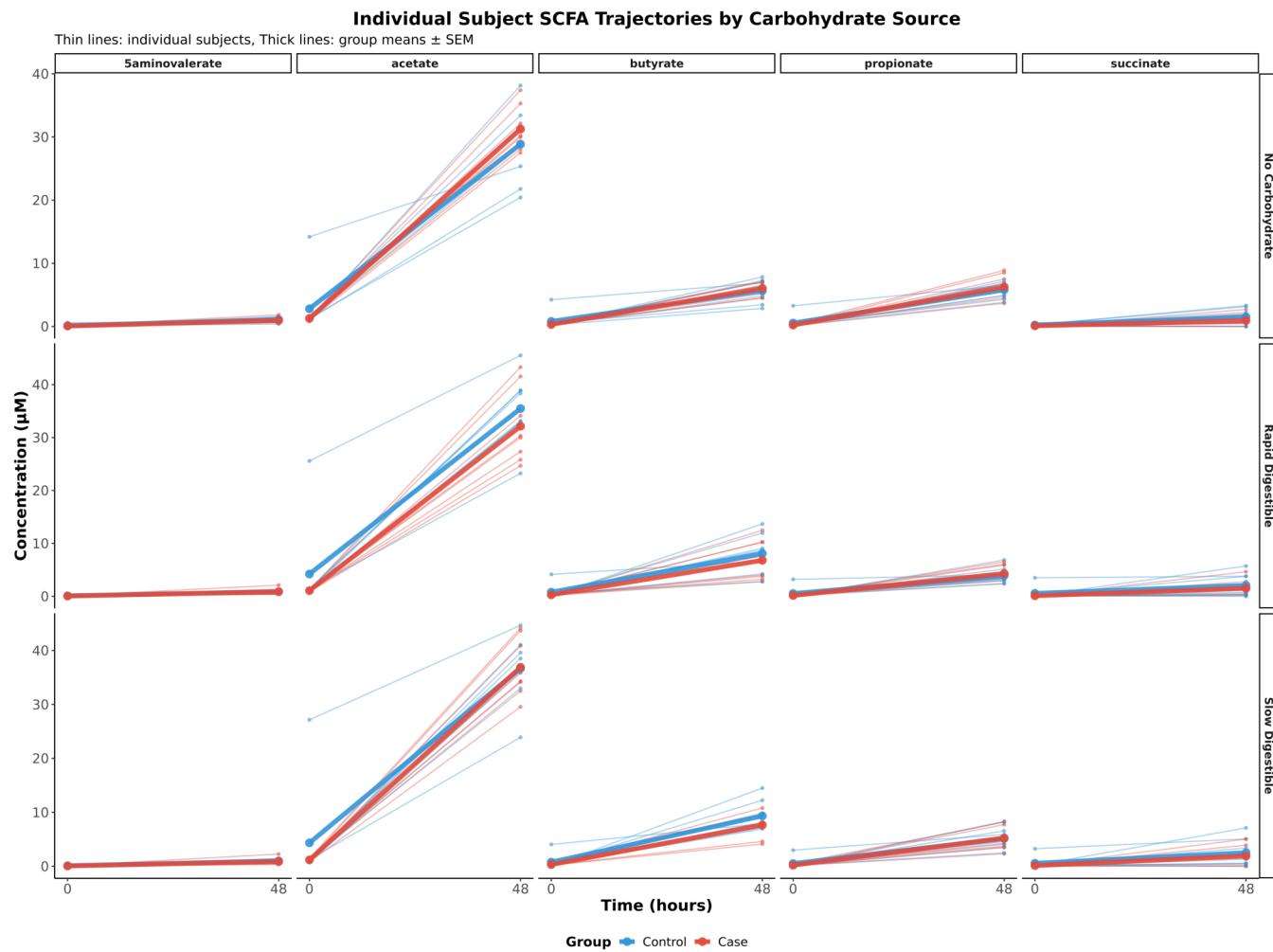
Interaction Effects



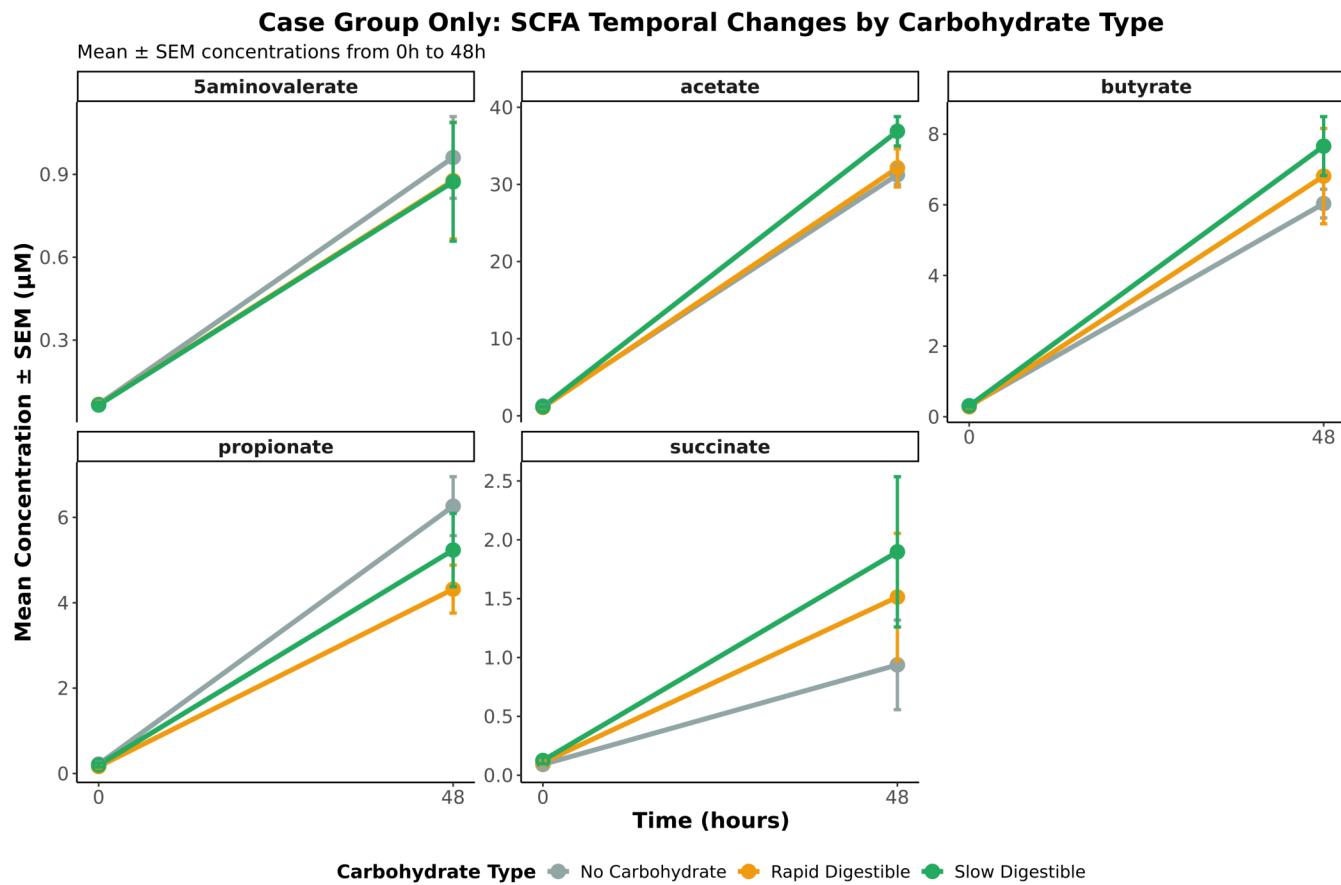
Subject-Level Individual Response Heatmap



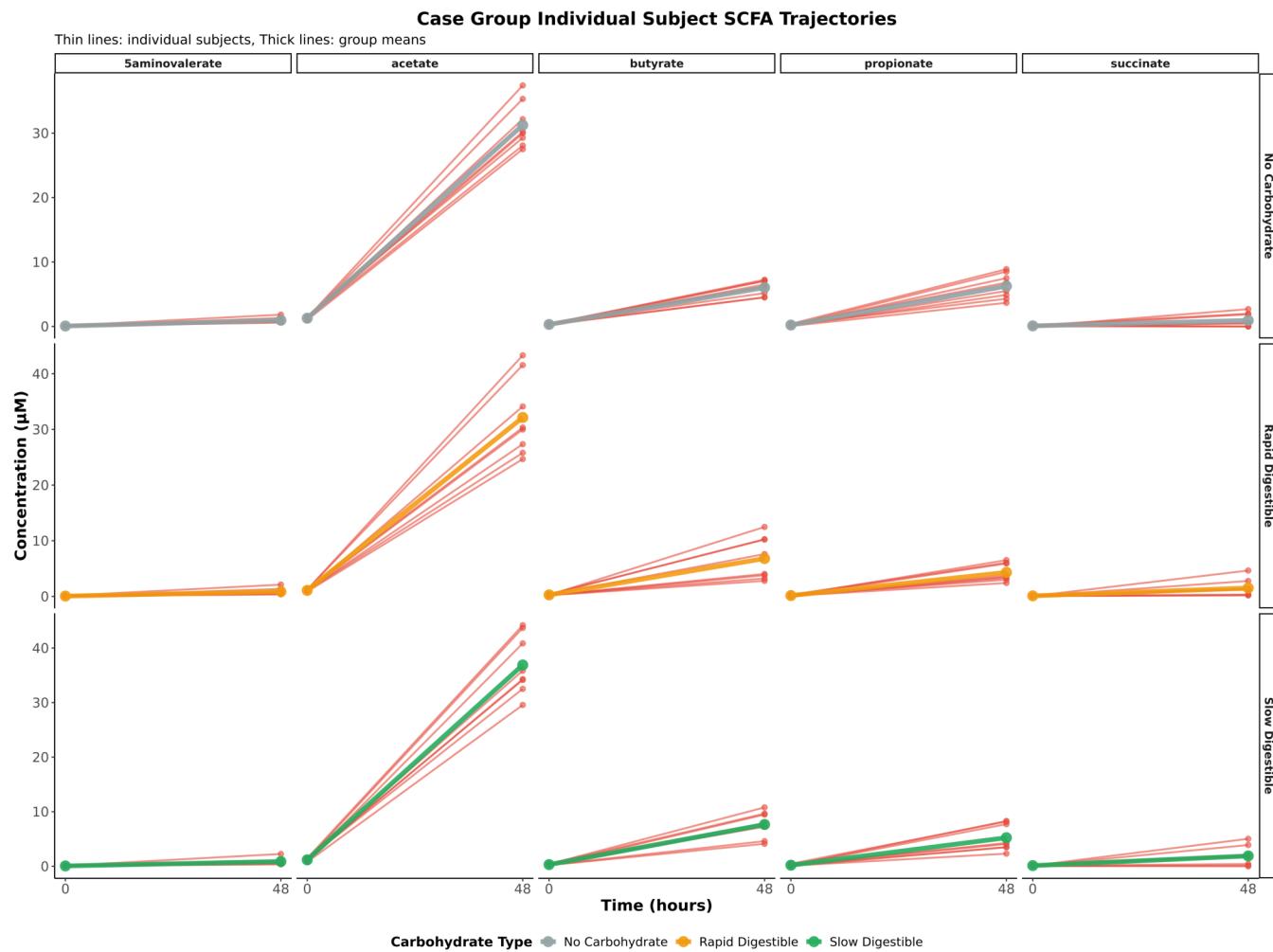
Individual Subject Trajectories by Carbohydrate Source



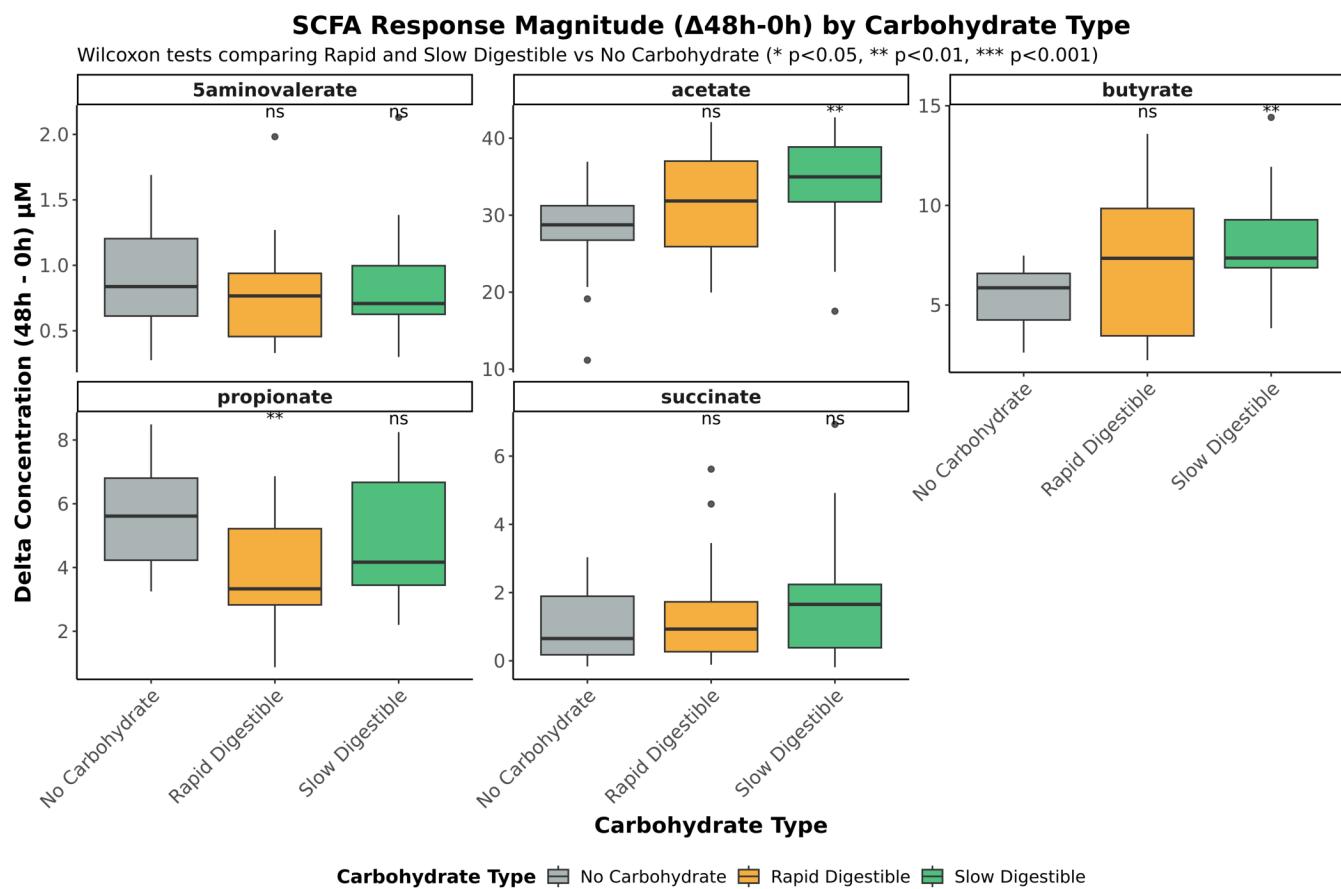
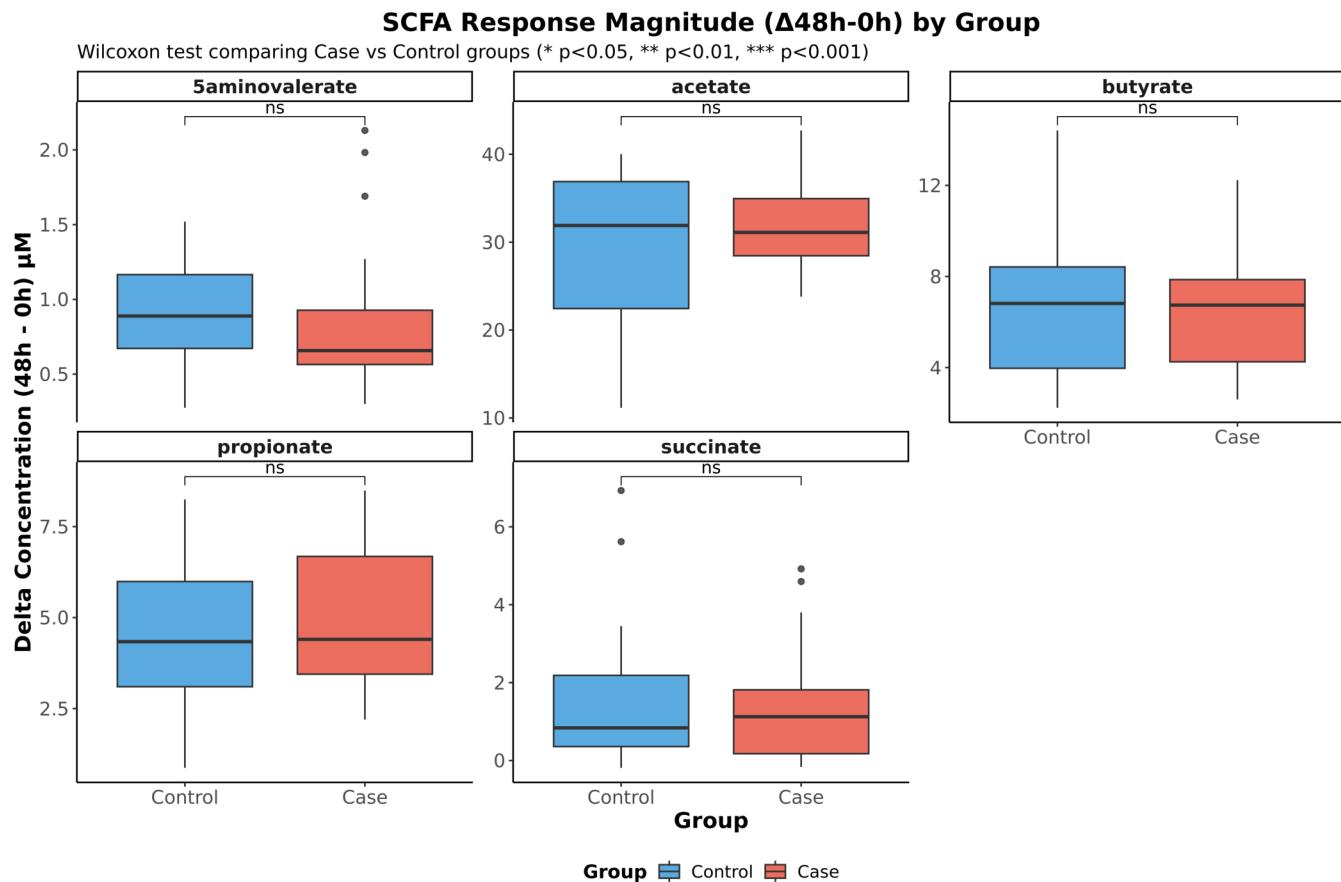
Case Group Only: Temporal Changes

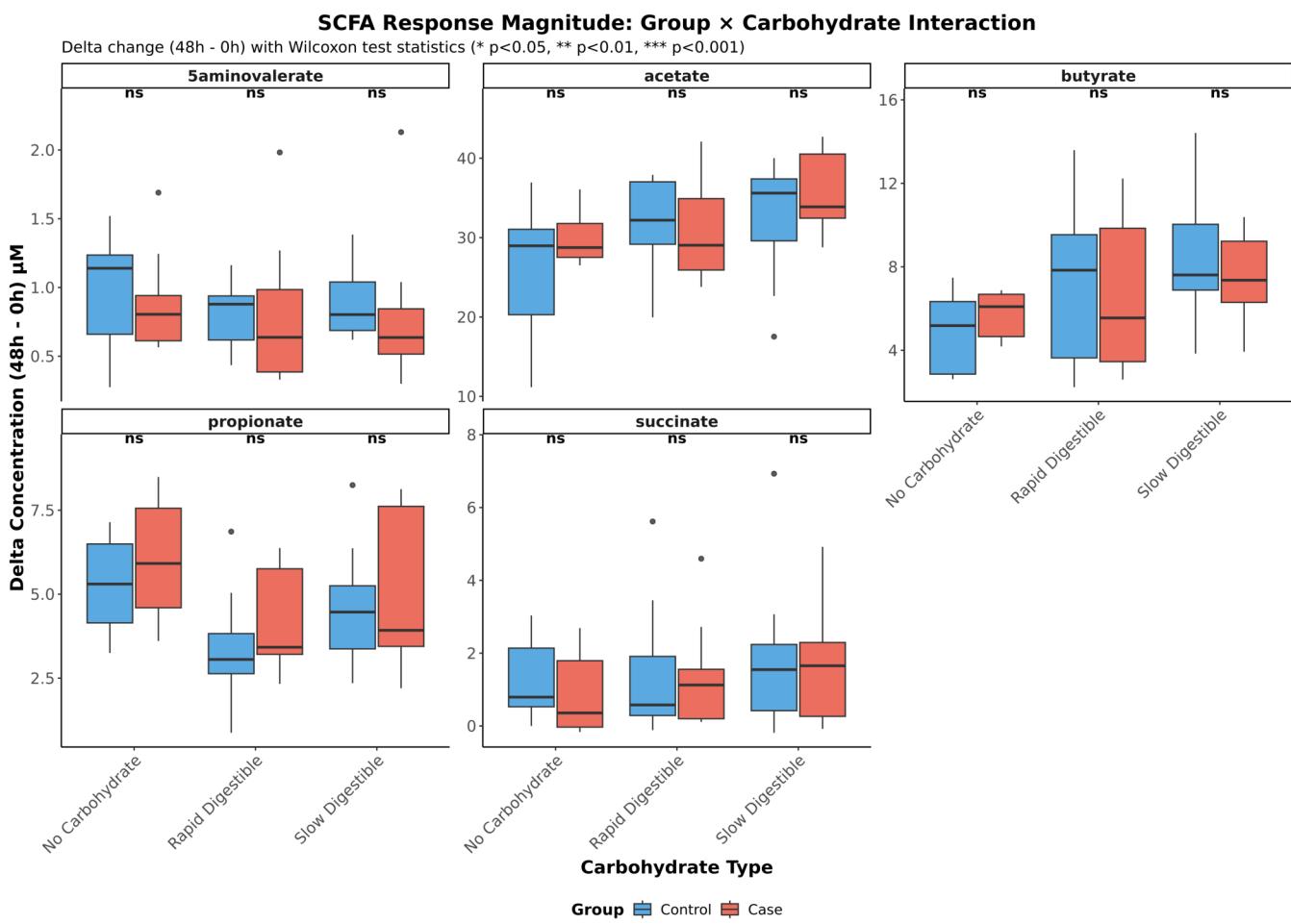


Case Group Individual Subject Trajectories



Delta Change (48h-0h) Visualizations





Save Plots

```
## All plots saved to plots/ directory with publication-quality formatting
```

Discussion

Key Statistical Findings

Temporal Effects Are Dominant

The most striking finding across all analyses is the **universal temporal effect** from 0h to 48h. Every SCFA analyte showed highly significant temporal changes ($p < 2e-16$ for all compounds), with dramatic increases from baseline to 48h:

- **Acetate:** $3.69 \mu\text{M} \rightarrow 34.0 \mu\text{M}$ (9-fold increase)
- **Butyrate:** $0.745 \mu\text{M} \rightarrow 7.38 \mu\text{M}$ (10-fold increase)
- **Propionate:** $0.502 \mu\text{M} \rightarrow 4.91 \mu\text{M}$ (10-fold increase)
- **5-aminovalerate:** $0.108 \mu\text{M} \rightarrow 0.924 \mu\text{M}$ (8.5-fold increase)
- **Succinate:** $0.444 \mu\text{M} \rightarrow 2.05 \mu\text{M}$ (4.6-fold increase)

Group Differences Are Not Significant

Contrary to initial expectations, **no significant differences** were found between control and case groups for any SCFA analyte (all $p > 0.05$). This suggests that the experimental intervention did not create distinct SCFA metabolic signatures between groups when controlling for other factors.

Carbohydrate-Specific Effects Are Present But Modest

Carbohydrate type showed significant effects for **acetate, butyrate, propionate, and succinate** in mixed-effects models ($p < 0.05$), but these effects were modest compared to temporal changes:

- **Succinate** showed the clearest carbohydrate response, with slow digestible carbohydrates producing higher concentrations than no carbohydrate ($p = 0.009$ in post-hoc testing)
- **Propionate and butyrate** showed significant carbohydrate \times time interactions, indicating that carbohydrate type influences the temporal response pattern

Case Group Analysis Reveals Universal Temporal Responses

The case-only mixed-effects analysis confirmed that **all SCFA analytes increase significantly over time** in case subjects (all $p < 2e-16$). This demonstrates that the temporal metabolic response is robust and consistent across individual subjects.

Carbohydrate-specific findings in case subjects: - **Acetate:** Modest carbohydrate effect ($p = 0.043$) - **Succinate:** Both temporal ($p < 2e-16$) and carbohydrate effects ($p = 0.018$), with marginal interaction ($p = 0.030$) - **Propionate:** Near-significant carbohydrate effect ($p = 0.082$) and interaction ($p = 0.058$)

Delta Change Analysis (Response Magnitude)

To complement the analysis of raw concentrations, we analyzed the delta (48h - 0h) values to focus specifically on the *magnitude* of the metabolic response. This approach helps to clarify whether the experimental factors (group, carbohydrate type) influence the *rate of change* in SCFA levels, independent of baseline concentrations.

- **Group Effects on Response Magnitude:** The analysis of delta values confirmed **no significant differences** in the magnitude of SCFA changes between control and case groups for any analyte (all adjusted $p > 0.05$). This reinforces that the experimental intervention did not create differential metabolic response magnitudes between groups.
- **Carbohydrate Effects on Response Magnitude:** The delta analysis revealed **significant carbohydrate effects** for three key SCFAs before multiple testing correction:
 - **Acetate:** $F(2,45) = 3.25$, $p = 0.048$, with slow digestible carbs showing significantly higher response magnitude vs no carbohydrate ($p = 0.048$ adjusted)
 - **Butyrate:** $F(2,45) = 3.40$, $p = 0.042$, with slow digestible carbs showing significantly higher response magnitude vs no carbohydrate ($p = 0.048$ adjusted)
 - **Propionate:** $F(2,45) = 4.22$, $p = 0.021$, with rapid digestible carbs showing significantly higher response magnitude vs no carbohydrate ($p = 0.048$ adjusted)
- **Specific Carbohydrate Comparisons:** Post-hoc pairwise tests revealed that **slow digestible carbohydrates** enhanced acetate and butyrate response magnitudes, while **rapid digestible carbohydrates** specifically enhanced propionate response magnitude compared to no carbohydrate controls.
- **Interaction Effects:** The two-way ANOVA on delta values showed no significant interaction between group and carbohydrate type for any analyte, confirming that carbohydrate effects on response magnitude were consistent across both control and case groups.

Individual Subject Variation

Subject-level analyses revealed substantial inter-individual variation in baseline SCFA levels, particularly in the case group (higher standard deviations for most analytes). The mixed-effects models properly accounted for this variation through random effects, strengthening the temporal effect findings.

Biological Interpretation

Microbial Fermentation Response

The dramatic 4-10 fold increases in SCFA concentrations from 0h to 48h likely represent **active microbial fermentation** of dietary carbohydrates in the gut. This temporal pattern suggests:

1. **Lag phase** (0h): Minimal baseline SCFA production
2. **Active fermentation** (48h): Peak metabolic activity producing substantial SCFA concentrations

Carbohydrate Source and Microbial Metabolic Pathways

The substrate-specific SCFA response patterns observed reflect distinct microbial metabolic pathways and community dynamics:

Slow Digestible Carbohydrates and Acetate/Butyrate Enhancement

Slow digestible carbohydrates (e.g., resistant starches, certain fibers) typically reach distal regions of the colon where they undergo fermentation by microbes specialized in producing acetate and especially butyrate. This explains the significant increase in acetate and butyrate response magnitudes when slow digestible carbohydrates are present.

Key microbial taxa involved include: - *Faecalibacterium prausnitzii* (major butyrate producer) - *Roseburia* species (butyrate and acetate producers) - *Eubacterium* species (butyrate producers)

These organisms thrive on complex carbohydrates and provide sustained fermentation, leading to the enhanced acetate and butyrate production observed.

Rapid Digestible Carbohydrates and Propionate Dynamics

Rapid digestible carbohydrates (e.g., glucose, sucrose) are consumed quickly, primarily in the proximal colon, resulting in different metabolic outcomes compared to slow digestible substrates.

The significant enhancement of **propionate production** with rapid digestible carbohydrates reflects several mechanisms:

1. **Propionate Pathway Specificity:** Propionate is mainly produced through three microbial pathways:

- Acrylate pathway
- Succinate pathway
- Propanediol pathway

These pathways are often utilized by organisms such as *Bacteroides*, *Veillonella*, and some *Prevotella* species.

2. **Substrate Utilization Patterns:** Rapid carbohydrate fermentation may favor taxa and metabolic pathways that specifically enhance propionate production, possibly through:

- Direct substrate channeling into propionate-producing pathways
- Altered cross-feeding networks that support propionate synthesis
- Competitive dynamics that favor propionate producers over other SCFA-producing communities

Community Dynamics and Substrate Competition

The differential SCFA profiles suggest that **microbial community structure and metabolic output are highly substrate-dependent**:

- **Slow digestible carbohydrates** selectively enhance communities producing acetate and butyrate, providing sustained fermentation in distal colon regions
- **Rapid digestible carbohydrates** create different selective pressures, favoring metabolic pathways that enhance propionate production while having minimal effects on acetate/butyrate relative to no carbohydrate controls

Control Comparison and Endogenous Substrate Utilization

In the **no carbohydrate condition**, baseline SCFA production likely reflects fermentation of endogenous substrates such as: - Mucins - Host-derived glycans - Residual dietary components

The addition of specific carbohydrate substrates shifts this metabolic baseline in substrate-specific directions, explaining the observed response magnitude differences.

Limited Treatment Differentiation

The absence of group differences suggests that the experimental intervention may not have sufficiently altered gut microbiome composition or metabolic function to create detectable SCFA signature differences within the 48-hour timeframe studied.

Conclusions

This comprehensive SCFA analysis using PFBBBr derivatization and GC-MS quantification reveals several key findings:

Primary Findings

1. **Universal Temporal Response:** All five SCFA analytes showed dramatic 4-10 fold increases from baseline (0h) to 48 hours, indicating robust gut microbial fermentation responses regardless of treatment group.
2. **No Treatment Group Differentiation:** Despite expectations, no significant differences were observed between control and case groups for any SCFA analyte, suggesting the experimental intervention did not create distinct metabolic signatures within the study timeframe.
3. **Significant Carbohydrate-Specific Response Magnitude Effects:** Delta analysis revealed specific carbohydrate effects on SCFA response magnitudes:
 - **Slow digestible carbohydrates** significantly enhanced acetate and butyrate response magnitudes ($p = 0.048$ adjusted)
 - **Rapid digestible carbohydrates** significantly enhanced propionate response magnitude ($p = 0.048$ adjusted)
 - These effects demonstrate substrate-specific microbial fermentation pathways
4. **Robust Case Group Responses:** Case-only analysis confirmed universal temporal increases across all SCFA analytes, with some analytes showing additional carbohydrate-dependent response patterns.
5. **Delta Analysis Reveals Carbohydrate Substrate Specificity:** The delta change analysis (48h - 0h) confirmed no group differences but revealed **significant substrate-specific effects:** slow digestible carbohydrates preferentially drive acetate and butyrate production, while rapid digestible carbohydrates preferentially enhance propionate production.

6. Substantial Individual Variation: Subject-level analyses revealed considerable inter-individual differences in SCFA production, properly accounted for through mixed-effects modeling.

Statistical Rigor

The analysis employed multiple complementary statistical approaches: - Mixed-effects models controlling for subject random effects - Multiple testing corrections (Benjamini-Hochberg) - Paired analyses for repeated measures design - Case-specific temporal analysis with proper statistical controls

Clinical Implications

These findings have important implications for precision nutrition approaches:

- **SCFA production responses are universal** across subjects regardless of treatment group, suggesting robust baseline fermentation capacity
- **Substrate-specific fermentation pathways exist:** slow digestible carbohydrates preferentially enhance acetate/butyrate (beneficial for gut health and systemic metabolism), while rapid digestible carbohydrates enhance propionate (important for gluconeogenesis regulation)
- **Targeted dietary interventions** could potentially be designed based on desired SCFA profiles: slow digestible carbs for enhanced butyrate production (anti-inflammatory effects), rapid digestible carbs for propionate production (metabolic regulation)
- **Individual metabolic variation** is substantial and should be considered in future study designs
- **Treatment effects may require longer observation periods** or different intervention strategies to overcome the dominant carbohydrate fermentation response

Study Limitations

- The 48-hour timeframe may be insufficient to detect treatment-specific microbiome changes
- Sample size may limit power to detect subtle group differences
- The dramatic temporal effects may mask smaller but clinically relevant group differences

This analysis provides a robust foundation for understanding gut microbiome SCFA production patterns and demonstrates the importance of temporal dynamics in metabolomic studies.

References

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