DISSERTATION

MASS SPECTROMETRY-BASED APPROACHES TO EVALUATE THE BIDIRECTIONAL RELATIONSHIP BETWEEN MICROBIAL COMMUNITIES AND METABOLITES IN FOOD PRODUCTS

Submitted by

Eva Grace Keohane

Department of Horticulture and Landscape Architecture

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Doctoral Committee:

Advisor: Jessica Prenni

Charlene Van Buiten
Corey Broeckling
Davina Rhodes

ABSTRACT

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The relationship between microbial communities and the metabolites they produce is highly complex and deeply influential in human health. Advanced analytical instrumentation, particularly mass spectrometry, is essential for gaining a systems level understanding of these interactions. This dissertation investigates the bidirectional relationship between microbiomes and microbial derived metabolites through the application of robust analytical tools, including mass spectrometry and 16S rRNA sequencing.

The three chapters of this dissertation explore microbial metabolite dynamics across clinical, *in vitro*, and *in vivo* contexts. Chapter One reviews current food-based clinical trials and emphasizes the need for more rigorous chemical characterization of dietary interventions, as analytical approaches applied to clinical biospecimens often far exceed those used for the foods themselves. Chapter Two examines how sourdough microbial communities shape the chemical composition of fermented foods, with the goal of leveraging this knowledge to intentionally design microbiomes that enhance nutritional functionality and shelf stability. Chapter Three evaluates the effects of the phytocannabinoids, CBD and CBG, on host metabolism and gut microbiome health in vivo. Together, this work highlights the necessity of standardized, high-resolution analytical techniques to better understand the complexity of microbial systems and their impact on human health.

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TABLE OF CONTENTS

INTRODUCTION

Characterizing the relationship between food, microbes, and health is a key research focus for understanding interindividual responses to diet. In doing so, this work also challenges the reductionist view of food as merely "good" or "bad," or as a sum of its calories, revealing instead the dynamic system that connects food to its biological and environmental origins. Food reflects the environment and processes from which it originates: climate, genetics, farming practices, and seasonality all contribute to its composition[1], [2], [3]. Appreciating this complexity allows us to view food not only as a source of nutrition but also as a product of cultural and socioeconomic influences. Similarly, human health is shaped by a myriad of factors including a combination of genetics, environment, physical activity, and diet [4], [5], [6]. Recognizing that both systems are highly variable underscores the need for robust scientific approaches to study the mechanisms linking microbes, food, and health. This dissertation bridges chemistry and microbiology through mass spectrometry-based omics and 16S rRNA sequencing to better understand how food composition and microbial communities interact to influence health outcomes.

Fermentation, the chemical break down of a substance by microorganisms, is an early form of food processing dating back to ancient civilization before the Middle Ages [7]. Evidence suggests the production of beer, wine, and cheese in Mesopotamia around 7000 BCE [8]. By approximately 4000 BCE, cereal grain fermentation to produce bread had emerged in ancient Egypt, and by 100 BCE, bakeries were common throughout Rome[9]. The mechanistic understanding of fermentation began to develop much later, following the advent of pasteurization by Louis Pasteur and the identification of *Lactococcus lactis* in milk by Sir John Lister in the mid to late 1800's [8]. Around the same period, Elie Metchnikoff attributed the

longevity of Bulgarian peasants to their consumption of fermented dairy products and discovered that lactic acid bacteria (LAB) produced compounds, referred to as “disinfectant bodies”, that promoted host health [10]. Since then, fermented foods have been recognized for their potential health benefits, although the mechanisms underlying these effects remain incompletely understood. While the use of fermentation dates back millennia, only with the advent of modern analytical technologies has it become possible to begin to mechanistically understand how microbial metabolism shapes food chemistry and human health.

One established idea by which fermented foods influence health is through interactions with the gut microbiome[11]. Fermented foods may contain live microbes that promote the growth of beneficial gut species, or, as in the case of sourdough bread, contain microbial metabolites that persist after microbial inactivation by heat[12]. These metabolites, often bioactive small molecules, can modulate gut microbial composition and activity[13]. The interactions between microbes and metabolites are highly complex and dynamic, requiring the use of robust, high-throughput analytical platforms to elucidate these relationships.

Metabolomics, the comprehensive study of small molecules within a biological system, enables such investigations. Most metabolomics workflows utilize mass spectrometry, and analytical tool that detects compounds based on their mass-to-charge ration, allowing for both untargeted profiling of metabolite patterns and targeted quantification of specific analytes. Mass spectrometry can also be used to study proteins and enzymes. In parallel, 16S rRNA sequencing provides a cost-effective means to characterize microbial community composition. While each of these techniques independently yields valuable insight, integrating them enables a mechanistic understanding of how microbial communities shape chemical environments and how bioactive compounds in food influence microbes.

These interactions are critical to human health. The gut microbiome contributes to digestion, immune function, mental health, and host metabolism[14], [15], [16]. Within the gut, microbes can biotransform dietary compounds that make their way to the large intestine which can improve bioavailability of these compounds. Understanding the bidirectional relationship between the chemical make-up of food and microbial ecology is therefore essential to understanding how diet impacts health. Integrating metabolomics, proteomics, and sequencing approaches provides a framework to study these processes at the molecular level. Despite advances in metabolomics, proteomics, and sequencing, there remains a lack of integrated studies that connect microbial composition, food chemistry, and functional health outcomes.

This dissertation employs mass spectrometry-based omics approaches to investigate the dynamic relationships between microbial communities, food chemical composition, and host health. Chapter One reviews the application of analytical techniques in clinical trials investigating nutritional interventions, with a focus on polyphenols and their influence on the gut microbiome. This work highlights the need to match the analytical rigor of nutritional intervention characterization with that of clinical outcome measurement to establish mechanistic links between dietary components, gut microbiome alterations, and health outcomes.

Building on the analytical framework discussed in Chapter One, Chapter Two applies these principles to a complex fermented food system exploring the influence of sourdough starter culture microbiomes on the chemical composition of breads and doughs. Untargeted metabolomics was utilized to characterize the chemical composition of breads and dough, and 16S rRNA sequencing was applied to identify microbial taxa across diverse starter cultures. The inflammatory potential of the resulting breads was further evaluated in an intestinal cell model. This work revealed distinct chemical profiles between sourdough and yeast-leavened breads,

demonstrated higher relative abundances of bioactive compounds in sourdough, and evaluated the correlation between chemical composition and predicted microbial functional capacity of sourdough starter cultures.

Extending from microbial-food chemistry interactions, Chapter Three explores how plant-derived bioactive compounds influence host–microbe interactions in-vivo. This work examines the sex-dependent effects of orally administered cannabidiol (CBD) and cannabigerol (CBG) in a murine model. Here mass spectrometry- based metabolomics was used to quantify phyto- and endocannabinoids in plasma and adipose tissue, while proteomic and microbiome analyses were applied to assess treatment effects on ileal function and microbial composition. Females exhibited higher circulating and tissue levels of phytocannabinoids compared to males. In males, CBD treatment was associated with increased plasma concentrations of the endocannabinoid anandamide (AEA), potentially linked to elevated levels of serine protease inhibitors identified in the proteomics data, which may reflect inhibition of the AEA-degrading enzyme fatty acid amide hydrolase (FAAH). Modest microbial taxonomic shifts were observed in the gut microbiome, although overall community structure remained stable. Collectively, these studies demonstrate the utility of integrating mass spectrometry-based metabolomics with genomic sequencing to elucidate microbial influences on chemical composition of foods and the effects of food-derived compounds on microbial communities in the context of health.

Diet related chronic diseases are the cause of death for one million Americans annually, and yet many of these conditions can be prevented or managed through dietary changes [17]. Although the connection between diet and health is well established, the field is still in its infancy when it comes to understanding the vast diversity of molecules in the food supply and how their structural variation collectively influences gut microbial communities. Advancing this

understanding will improve our ability to optimize dietary interventions for human health. Future progress will require interdisciplinary collaboration across analytical chemistry, clinical and nutrition research, and food science to comprehensively characterize food products, elucidate their metabolic fate, and define their physiological impacts. There is also a critical need to characterize microbial-derived byproducts of fermented foods and to connect dietary inputs with their metabolic transformations in the body processes that are likely influenced by individual differences in host and microbiome composition.

As demonstrated in Chapter One, few clinical studies have incorporated untargeted metabolomics of nutritional interventions. Expanding such analyses could provide mechanistic insight into individual variability in dietary response and aid in developing precision nutrition strategies to mitigate chronic disease. Building from in-vitro and in-vivo research models, my future work will extend toward clinical investigations to further elucidate the complex interactions between polyphenols, microbial metabolism, and human health.

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CHAPTER 1- COMPARING APPLES TO APPLES: EVALUATING THE USE OF FOODOMICS IN PRECISION NUTRITION RESEARCH

Abstract

This review aimed to evaluate the use of advanced omics methodologies in dietary intervention clinical trials focused on evaluating the influence of polyphenols on the gut microbiome. All published clinical studies in the Cochrane Library database from 2014 to 2024 containing the words “polyphenols” and “gut microbiome” were compiled and categorized based on experimental design, analytical methodologies and findings. We found that despite known inconsistencies in food composition across agricultural and processing parameters, omics analysis of the food used in interventions has not been widely embraced. None of the studies evaluated employed untargeted omics approaches for food composition analysis, while 5 of the 38 studies did use untargeted omics for clinical samples analysis. Targeted analytical methods focused on known compounds or proxies were more commonly used for food composition analysis (18 of 38 studies) and clinical samples (24 of 38 studies), though analysis of clinical samples focused on a greater number of target compounds. Data from these studies support

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Introduction

Precision nutrition is an emerging scientific approach aimed towards optimizing and tailoring dietary guidance with specific recommendations based on shared characteristics of a demographic, such as sex, age, genetics, metabolism and environmental factors [1]. The identification of shared risk profiles enable targeted or "precision" dietary approaches to mitigate disease risk and support human health [2,3]. In support of this approach, clinical nutrition research has adopted advanced analytical techniques that allow for characterization of genome and microbiome variations that influence digestion, uptake, metabolism, excretion, and biological effects of food nutrients and metabolites [3]. These high-resolution analyses have revealed the importance of identifying and acknowledging inter-individual variability in nutrition research, which is widely acknowledged as a pathway to more effective dietary guidance.

In recent years, similar high-resolution analytical approaches have also been applied to the chemical characterization of food. The field of "foodomics" combines food chemistry, biological sciences and informatics through the application of omics technologies (e.g., metabolomics, lipidomics, proteomics) to comprehensively characterize the molecular composition of food, identifying a broad spectrum of known and unknown compounds and providing information about their relative abundances in a given food sample. Though the food metabolome was once considered as merely a component of the human metabolome that is derived from digestion and biotransformation of the foods and their components (**Table 1**) [4], recent advancements in food analysis research have revealed unique metabolomic profiles within foods themselves, influenced by agricultural and processing conditions and unrelated to human digestion [5,6].

Table 1 Key terms relevant to the study of metabolites.

Term	Definition
metabolite	intermediate or end product of metabolism, produced by any living tissue (e.g., short chain fatty acids produced by bacteria, phenolics produced by plants, neurotransmitters produced by animals); primary metabolites are directly involved in growth, development and reproduction while secondary metabolites mediate ecological interactions (e.g., defense) [7]
metabolome	the complete set of metabolites within a given biological system at a given time [7]
metabolomics	comprehensive measurement of all metabolites in a biological system [7]
foodomics	the application of untargeted omics technologies (e.g., metabolomics, lipidomics, proteomics) to comprehensively characterize the molecular composition of food, identifying a broad spectrum of known and unknown compounds and provide information about their relative quantities in a given food sample [5]

For example, metabolomic analyses comparing wild and domesticated apple cultivars has demonstrated significant differences in phenolic compounds often associated with health including tannins, flavonoids and phenolic acids [8]. Beyond variability among food components with known bioactivity, untargeted foodomics approaches have also revealed numerous food components present in foods that have yet to be identified, referred to as "nutritional dark matter" [9].

This newly realized molecular complexity of food reveals previously unrealized challenges in the efforts to generate specific dietary recommendations. Since 2009, publications focused on "precision nutrition" have outpaced those associated with "foodomics" (**Figure 1**), suggesting a potential gap between these two areas of research that may hinder advances in precision nutrition. Polyphenols, for example, are a diverse class of phytochemicals that have been shown to differentially modulate gut microbial composition and function depending on their chemical structure, degree of polymerization and integration into different food matrices [10]. Thus, if "an

apple a day keeps the doctor away", modern analytical approaches suggest that not all apples may be equally proficient in supporting this goal based on differences in chemical composition.

The overarching objective of this evidence-based review was to examine existing practices in characterization of food composition in clinical nutrition research. To conduct a focused and interpretable review of analytical methods, we limited our scope to human intervention trials investigating the impact of polyphenols on the gut microbiome. This research area was chosen because polyphenols have been shown to be highly variable across food sources [11–13] and the microbiome has been noted as a major source of interindividual variability in clinical studies and a cornerstone of precision nutrition research [14–18]. Through this lens, we systematically examine and compare the reported analytical approaches for characterization of the food interventions administered to research participants as well as those used to analyze the clinical biological samples collected in a collection of clinical trials from 2014 to 2024. Our findings highlight the variety of approaches applied to food composition analysis on the "front-end" of clinical trials and underscore the need for more robust and comprehensive characterization of the food interventions in clinical studies with an end goal of improving human health and supporting advancements in precision nutrition.

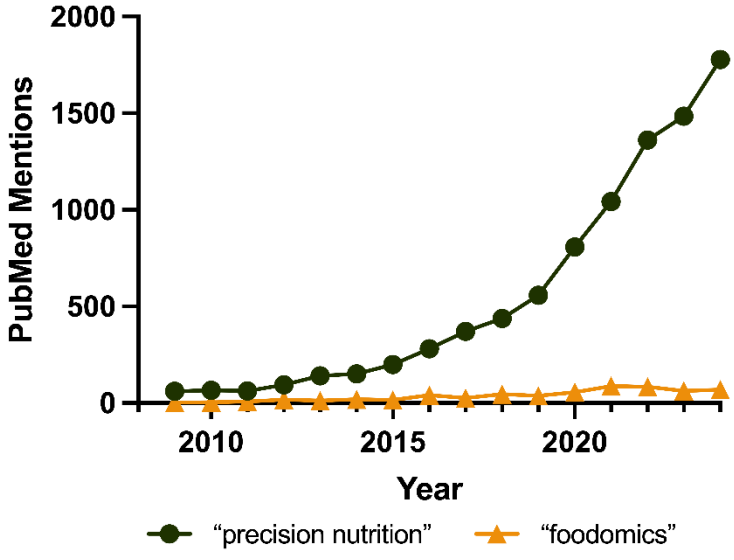

Figure 1 Research into precision nutrition has outpaced comprehensive nutrient characterization of food (e.g., "foodomics"). Data retrieved from PubMed on 28 January 2025.

Materials and Methods

This review aimed to characterize analytical methods currently used to study polyphenols and polyphenol-derived metabolites in dietary interventions and in biological samples collected from clinical research participants in studies investigating the relationship between dietary polyphenols and the gut microbiome. First, we identified all registered clinical studies in the Cochrane Library database from 1 January 2014 to 27 September 2024 with the key words "polyphenol" and "gut microbiome". The Cochrane Library database was chosen for this systematic review due to its specialized focus on clinical trials and comprehensive indexing, allowing for improved methodological efficiency. In total, the database search showed 124 results.. The following inclusion criteria were applied to refine the dataset: (i) dietary intervention studies for humans focused on polyphenols in the form of polyphenol supplements, polyphenol-rich foods or a polyphenol-enriched diet; (ii) studies with keywords "microbiome" or "microbial diversity"; (iii) studies published from 1 January 2014 to 27 September 2024; (iv) original research articles. The exclusion criteria were: (i) studies performed in animal models or *in vitro*; (ii) conference proceedings, clinical trial registry records, letters to the editor, opinion pieces, review articles. After analysis by two independent referees, 42 publications that met these criteria were included (**Figure 2**). Research not meeting inclusion criteria was used to provide context and support where needed.

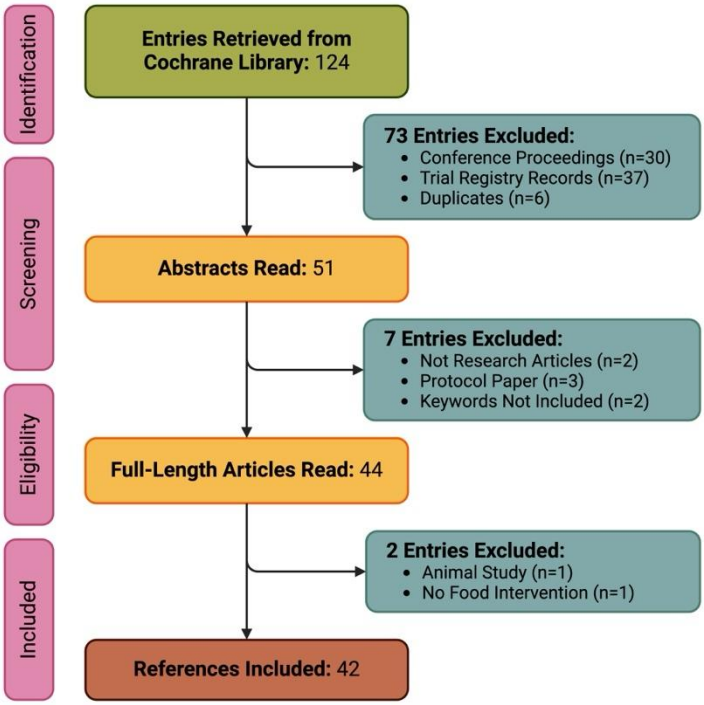

Figure 2 Flow diagram of literature search and selection process. References containing the words "polyphenols" and "gut microbiome" were retrieved from the Cochrane Library. Exclusion criteria included conference proceedings, clinical trial registry records, non-research articles (e.g., literature reviews, letters to editor) and protocol papers. Inclusion criteria included clinical trials with human subjects, food interventions and identification of keyword "microbiome" or similar (e.g., microbial diversity). Image generated using BioRender (https://BioRender.com).

Results & Discussion

As illustrated in **Figure 2**, 124 references were identified in the Cochrane Library Database and accessed from Google Scholar and PubMed. Of these references, a total of 42 papers representing 38 clinical trials were ultimately determined to meet inclusion criteria. Two large-scale studies were represented by three papers each. **Table 2** summarizes the characteristics of the clinical trials covered by the reference articles. Of the 38 clinical trials, 4 analyzed the influence of polyphenols on the gut microbiome within the context of the whole diet, while 19

focused on a single food (e.g., blueberries) and 15 focused on a dietary supplement or experimental food (e.g., pomegranate extract, olive pomace-enriched biscuit). Study sample sizes ranged from 10 to 1874 participants, though most studies included 50 or fewer participants. Healthy adults were the predominant study population, but individuals with metabolic syndrome, obesity and various cardiovascular disease risk factors were also noted across study populations. Studies lasting 1 – 4 weeks were most common, but intervention periods ranged from acute studies of one day (a single dose) to chronic interventions up to 18 months. Nearly all (92.1%) studies collected blood from participants, and a majority (84.2%) of studies also collected stool/fecal samples. Other biological samples included urine, saliva and skeletal muscle. Only one study did not collect any biological material and instead used information from a publicly available data set [19].

Table 2 Description of study characteristics.

Characteristics	***N***
Intervention Type	
Whole Diet	4
Single Food Intervention	19
Supplement/Experimental Food	15
Population Sample Size	
< 25	10
26 – 50	15
51 – 100	11
> 100	2
Population	
Children	0
Adults	36
Elderly	2
Gender	
Men Only	5
Women Only	3
All Genders	30
Health Status	
Healthy	19
Metabolic Syndrome	7

Overweight or Obesity	7
Cardiovascular Disease Risk Factors (General)	6
Other	1
Study Length	
Single Dose	3
1 – 4 weeks	15
5 – 8 weeks	10
9 – 12 weeks	9
> 12 weeks	3
Biological Sample Collection	
Blood	35
Stool	32
Urine	15
Saliva	1
Skeletal Muscle Biopsy	1
None	1

Summary of Influence of Polyphenols on the Gut Microbiome

The overarching findings from the subset of clinical trials examined in this review underscore the challenges associated with interpreting clinical findings related to polyphenols and microbiome. While all but four manuscripts reported some influence of polyphenol intake on the microbiome, the effects measured varied widely and were, in some cases, contradictory.

Across interventions, many trials reported no significant changes in alpha or beta diversity [20–35], while others reported increases in diversity as reported by Shannon, Chao1 or gene richness [19,36–38]. Notably, inconsistencies existed even in studies with the same intervention food [19–21,36], suggesting that the specific composition of polyphenols may influence these discrepancies, as well as the food matrix in which the polyphenols are delivered.

Similar inconsistencies across findings were observed with regard to taxonomic shifts. In the seven studies that characterized the influence of the intervention on *Lactobacillus*, a significant increase was reported in five studies, while no change was reported in the other two [23,25,27,39–42]. Examination of the relative abundance of other classes of organisms including

Bifidobacterium, *Faecalibacterium*, *Prevotella*, *Roseburia* and *Ruminococcus* revealed results that were directly conflicting, with some studies reporting significant increases and other reporting significant decreases [19,25–30,32–34,39–46].

Some of the discrepancies demonstrated in this data set can be attributed to differences in participant population, as demonstrated by studies comparing the influence of intervention on healthy subjects versus a disease state. This highlights the benefit of characterizing details about study participants with respect to factors like genetics, metabolism, and environmental factors, which are key areas of consideration in precision nutrition. However, the absence of consistently robust compositional characterization of the polyphenols found in the intervention foods across all trials, as described in the following sections, limits the ability to evaluate the extent to which differences in outcomes may also be attributed to the dietary interventions.

Food Composition Analyses

Food composition can vary on the basis of geography, agricultural practices, storage and processing [8,47–50]. When investigating the effect of a food or specific component of a food on a health-related outcome, compositional characterization of the food in question can be used to validate the experimental design. These analyses focus on the quantification of parent compounds, or those original, unmodified chemical compounds present in the food, though the level of specificity in terms of identifying and quantifying unique compounds varies by method. The papers included in this review used a range of methods for compositional characterization including public databases, nonspecific methods (e.g., those measuring total phenolics) and targeted analytical methods. Descriptions of these overarching classifications of characterization

methods are listed in **Table 3**. Frequency of use of each method in characterizing both food composition and clinical samples is depicted in **Figure 3**.

Table 3 Compositional analysis of foods and clinical samples.

Type of Analysis	*N* [†]	Total # of metabolites	Description	Advantages	Limitations
Database	7	N/A	Compilation of compositional information from published literature	Free of cost, no laboratory equipment required [100]	Often inaccurate due to natural diversity in foods (e.g., geographical region, cultivar, processing), lack of standardized analytical procedures, and limited foods analyzed [100]
Non-Specific Methods					
Food Composition	1 2	N/A	Quantitation of a total class of compounds using a non-specific outcome (e.g., total polyphenols by reducing activity)	Relatively simple and inexpensive [101]	Subject to interference by compounds with similar activities, does not provide compositional information [101]
Clinical Samples	1	N/A			
Targeted Analytical Methods					
Food Composition	1 8	250 (Range: 2-46)	Validation and absolute quantification of specific known molecules in comparison to	Highly specific, accurate and efficient [102]	Limited coverage, susceptible to bias, expensive [103]
Clinical Samples	2 4	1556 (Range: 2-450)			

			internal standards		
Untargeted Analytical Methods					
Food Composition	0	N/A	Comprehensive analysis of all small molecules with relative quantification	Comprehensive coverage allows discovery of novel compounds and system-level insights [104]	Complex data analysis and interpretation [103,105]
Clinical Samples	5	25,438 (Range: 23-24,956)			

† = Table generated from 38 studies. 10 studies used more than one method of compositional analysis; 4 studies did not report any compositional information; 6 studies reported compositional information without reference to the methods used to obtain these results.

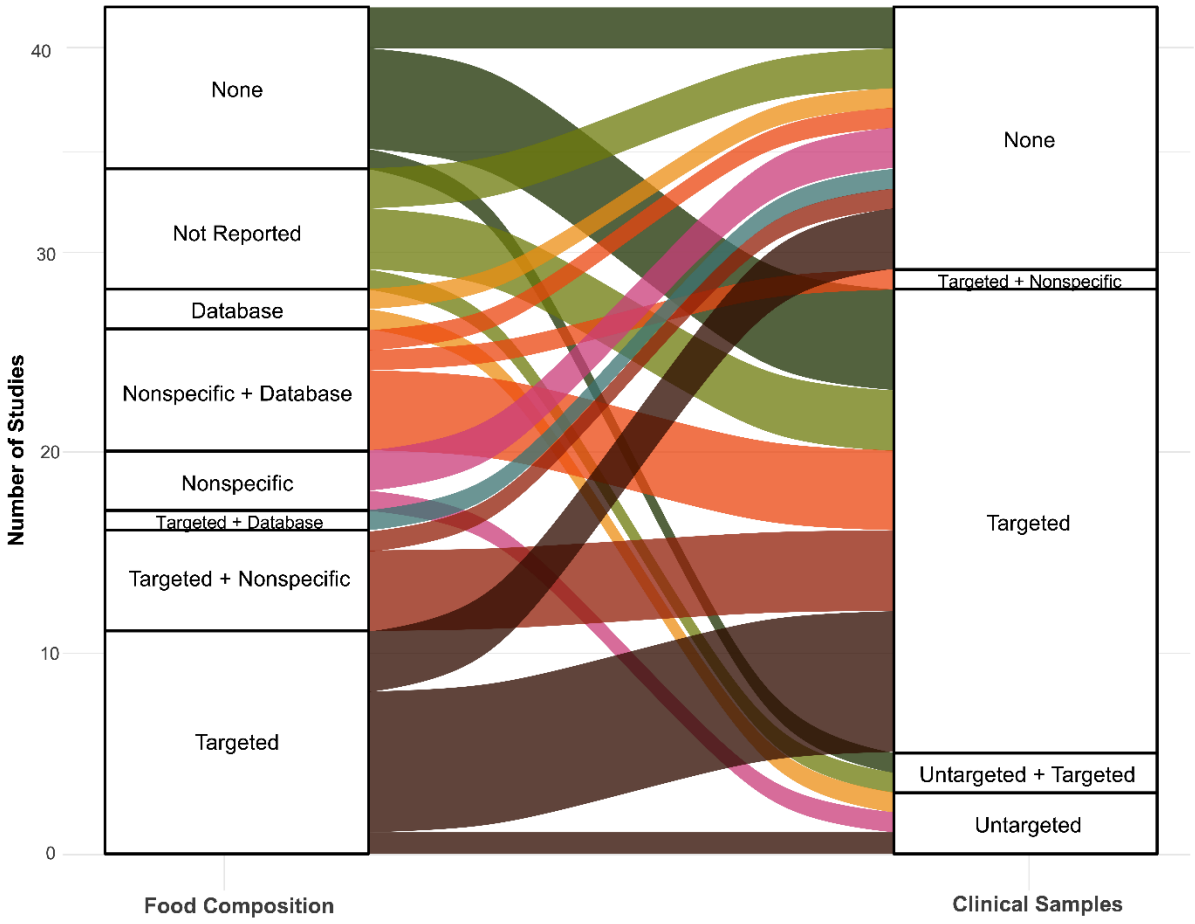

Figure 3 Frequency of food composition analysis methods and corresponding clinical sample analysis method from the queried studies. Food composition analyses included none, not reported, database, non-specific, targeted and combinations of analyses. Clinical analyses included none, non-specific, targeted, untargeted and combinations of analyses.

No Phenolic Characterization

Six studies did not include any type of phenolic characterization of the dietary intervention used. The study food products used for these interventions included supplements/experimental foods (n=3), a single food (n=2) and a whole diet intervention (n=1). In the case of the whole diet intervention study, a "healthy diet" was used as a control in comparison to a Mediterranean diet and a plant-based Mediterranean diet [43]. The experimental plant-based Mediterranean diet included an increased number of foods typically rich in polyphenols including nuts, green tea and a green shake supplemented with *Wolffia globosa.* However, the lack of comprehensive polyphenol characterization in this study limited the potential for elucidation of possible mechanisms driving the observed changes in the abundance of *Bifidobacterium* and *Prevotella* in the gut microbiome of the participants [43].

Three of the six studies that did not include any method of polyphenol characterization also did not include metabolomic analysis of the clinical biological samples collected, suggesting a research focus specific to functional clinical outcomes. However, the addition of molecular characterization of the food intervention may provide valuable data to increase the precision and translatability of the findings related to host responses. One study examined the impact of 8 weeks of daily almond intake on the gut microbiome of healthy young adults and attributed observed increases in alpha diversity and decreases in abundance of *Bacteroides fragilis* in part to polyphenols found in almond skin [37]. Though the macronutrient profile of the dry-roasted almonds was provided by a database [57], processing techniques like roasting have been shown

to modify the phenolic composition of almonds, increasing concentrations of individual flavonoids such as quercetin, kaempferol, protocatechuic acid and *p*-hydroxybenzoic acid, but decreasing overall total phenolics, suggesting a loss of proanthocyanidins [58]. Similarly, almond processing has been shown to affect nutrient accessibility [59]. Given these differences, compositional analysis of intervention foods could help explain why roasted almonds influence microbial diversity in the gut of health adults in one study [37] but have no effect on diversity in another [60].

Public Databases

Seven studies used public databases for phenolic characterization of food interventions including interventions of the whole diet (n=4) and single foods (n=3). Three of the four whole diet interventions included at least one additional analysis of total polyphenols in at least one of the intervention foods. Of the three single-food interventions, a study examining the effects of mixed spices quantified total polyphenols in addition to using database estimation [25] while the studies examining the effects of mixed nuts and beer used publicly accessible databases to estimate levels of ellagitannin and total polyphenols, respectively [33,61]. The databases used included the USDA Database for the Flavonoid Content of Selected Foods [62] and Phenol Explorer [63]. The USDA database provides concentrations of 26 phenolic compounds in 506 foods, while Phenol Explorer provides information about the concentrations of 500 polyphenols in over 400 foods. Each database includes values for raw, unprocessed foods as well as some foods which have undergone significant processing including canning, drying and freezing.

Public databases such as those provided by USDA and Phenol Explorer can be valuable in instances where comprehensive analysis of food or diet during the interventions would be

impossible. For example, this may be true in the case where a group of participants were given dietary recommendations and asked to report their dietary intake through food records or food frequency questionnaires rather than having specific foods or meals provided to them. However, the fact that four out of seven studies combined their own phenolic analysis with database estimations underscores the level of caution that should be used if relying solely upon database estimations in clinical research. There are limitations associated with the use of such databases. For example, in each database, the concentrations of phenolics reported for each food product have been extracted from published literature. Although the data included were evaluated for quality and acceptable methodology, there are a variety of factors that can limit the translatability of information gleaned from databases. Notably, the USDA database does not differentiate on the basis of cultivar in all plant foods [62]. Beyond this, as previously discussed, a variety of factors not necessarily catalogued by databases can influence nutrient composition including, but not limited to, geographic region grown, agricultural practices, harvest and post-harvest practices and processing [64].

Nonspecific Methods of Phenolic Characterization

Nonspecific phenolic characterization techniques are often relied upon for their ease of implementation, low cost, and ability to be quickly interpreted. Within the context of this review, nonspecific methods of phenolic characterization refer to primarily colorimetric assays for the quantitation of the total amount of large classes of phenolic compounds (e.g., total polyphenols, total anthocyanins). Of the reviewed studies, 12 used nonspecific methods of phenolic characterization. These assays were often used in conjunction with other compositional analyses; for example, three studies quantified total polyphenols in some foods while using a public

database for others, while six studies combined nonspecific methods with targeted analytical methods.

The most employed nonspecific method used in this cohort of studies was the Folin-Ciocalteu method for quantifying total polyphenol contents, which was used in all 11 of the studies featuring nonspecific analyses. Similar nonspecific spectrophotometric methods also exist for measuring total flavonoids, total anthocyanins and total proanthocyanidins . Though useful in determining baseline values of phenolic compounds, these methods are not without limitations. For example, the absence of chromatographic separation yields less sensitive and less specific results which may be susceptible to interference from complex food matrices. Additionally, calculation of total amounts of a class of compounds does not take compositional variation into account, thus overlooking potentially important differences in functionality. As previously discussed, structural variability in polyphenol composition can lead to differences in bioavailability, solubility, protein binding and enzyme inhibition, each contributing to potential overarching differences in human biological functionality [68,69].

Though valuable information can be obtained from dietary intervention clinical studies even in the absence of detailed food compositional data, reliance on nonspecific methods of total phenolic characterization can limit the interpretation of study results. For example, a study examining the effect of Montmorency (tart) cherry juice on the gut microbiome and markers of metabolic health in middle-aged individuals with obesity found that the tart cherry juice did not affect serum insulin concentration, the gut microbiome or inflammatory markers including interleukin-6 and C-reactive protein [70], despite this effect being reported by others studying Montmorency cherry and other tart cherry supplements [71–74]. It was hypothesized that this discrepancy in results may be due to differences in the baseline inflammation levels of the study

participants [70], but the impact of specific phenolic composition cannot be ruled out. Indeed, targeted mass spectrometry analysis of juice from five different cultivars of tart cherries, including Montmorency cherries, has demonstrated that phenolic composition of cherry juice is influenced by cultivar, supplement processing method and storage [75,76]. Thus, it is possible that compositional differences between interventions may drive variation in efficacy observed across clinical trials.

Targeted Analysis

Targeted analyses are focused and quantitative approaches to characterizing the chemical composition of a sample based on purified standards. These techniques typically employ a separation technique (e.g., liquid chromatography) followed by a sensitive detection method (e.g., mass spectrometry, photodiode array). The sensitivity and specificity of targeted approaches make them valuable for the quantitation of known biomarkers and compositional analysis of commonly studied products. However, the pre-selection of analytes can lead to limited coverage and possible bias due to incomplete characterization of the sample. Targeted analysis was the most frequently applied method of analysis for phenolic characterization of the foods used in the clinical trials evaluated. Of the 18 studies that featured this approach, six used it to complement nonspecific analyses and one used it in conjunction with a database. The number of polyphenols identified in each study ranged from 2 to 46 with a median of 12.

As previously described, compositional characterization of the phenolic composition of food interventions used in clinical trials has the potential to allow better informed comparisons of results across studies using similar food products. We identified two clinical trials examining the effect of red wine on the gut microbiome. In one study, 250 mL/day of Pinot noir was given to

15 healthy adults for 4 weeks [77,78] and in the other, 272 mL/day of an undisclosed varietal or a dealcoholized equivalent was given to 20 adults (10 healthy, 10 with metabolic syndrome) for 2 weeks each [39]. The health effects of red wine have been studied widely and are most frequently attributed to its high phenolic content [79,80], but the overall phenolic composition of red wine can vary due to differences in varietal, vintage, style, and packaging [81,82]. In the study using a 2010 Pinot noir as a treatment, 46 phenolic compounds were quantified in the wine using liquid chromatography with mass spectrometry detection [77,78]. Despite the treatment in the second study being referred to only as "red wine" with no information about varietal or vintage, the quantification of 26 different phenolic compounds using liquid chromatography with spectrophotometric detection [39] enabled comparison of the studies and revealed 20 overlapping phenolic compounds with known bioactivity. In both studies, diversity of the gut microbiome was increased as a result of the red wine intervention; in healthy adults, diversity increased from baseline [78] and baseline differences in diversity between healthy adults and those with metabolic syndrome were reversed after the intervention [39]. In this example, the use of targeted approaches enabled observation of compositional overlap in the phenolic compounds across the two studies, however, because the targeted panels and instrumentation used in each study were different, we are still left with uncertainty about the influence of the non-overlapping compounds (detected in only one study) and the broader complement of phenolic compounds not prioritized in the targeted analysis.

As a whole, the studies which included compositional characterization of food interventions identified 146 unique metabolites in food (**Figure 4A**). When classified into overarching categories of metabolites, flavonoids and hydroxycinnamic acids comprised the greatest number of unique analytes characterized in foods (**Figure 4B**), while flavonoids, benzoic acids and

hydroxycinnamic acids were the most frequently characterized classes of compounds. The most frequently identified individual compounds in food samples were epicatechin, caffeic acid, protocatechuic acid, gallic acid and catechin (**Figure 4D**).

The identification of parent compounds in clinical interventions can provide valuable mechanistic insight towards the effect of food composition on the gut microbiome and related clinical outcomes. This is particularly important in the case of dietary polyphenols, which tend to be poorly absorbed in the gastrointestinal tract on the basis of solubility and stability [83] and instead exert local effects in the gut (e.g., prebiotic activity, antioxidant activity) [84,85] or become metabolized by the gut microbiota into short-chain fatty acids or other active metabolites [86]. When combined with characterization of metabolites from clinical samples (e.g., blood, urine), information regarding parent compounds found in food products has the potential to be used to predict health-associated outcomes and thus enhance the accuracy and precision of precision nutrition approaches.

Untargeted Analysis

Untargeted analyses employ the similar instrumentation as targeted analysis but use these techniques to instead qualitatively characterize the chemical composition of a sample without reliance on purified standards. The comprehensive nature of untargeted techniques are valuable for exploring a broad metabolite profile without prior assumptions about its composition. However, one of the major challenges of food composition analysis is the vast chemical diversity present in food which requires multiple analytical methods for comprehensive analysis. Thus, even untargeted methods will be biased for the types of molecules they can detect based on analytical variables (e.g., method of extraction, type of chromatography, type of detector, etc.).

Despite the potential for untargeted analysis to profile a wide range of compounds for foods in clinical trials, none of the studies queried included untargeted analysis of foods.

Metabolite Analyses of Clinical Samples

Both targeted and untargeted metabolite analyses are commonly performed in clinical trials for the quantification of food-associated metabolites in biological samples and biomarkers associated with health and disease. In the case of studies focused on the effect of dietary interventions on the gut microbiome, these analyses can provide useful insight towards the processing of parent compounds from food by the gut microbiota [87]. Metabolite analysis of clinical samples was reported in 29 of 42 papers (26 of 38 studies). The number of metabolites reported in a single study ranged from 2 to 450 (median = 19) in studies employing targeted approaches and 19 to 24,956 in those employing an untargeted approach (median = 232), highlighting an imbalance in detected metabolites in clinical trials based on the analysis used. Only one study used a nonspecific method of phenolic analysis in their clinical samples, quantifying total polyphenols in urine based on the Folin-Ciocalteu method, though this was combined with a targeted analysis of phenolic acids in plasma [25].

Targeted Analysis for Clinical Samples

Targeted analyses were the most prevalent methods applied to clinical samples, featured in 24 of 42 papers included in this review. These analyses were most frequently applied to plasma samples (17 studies), followed by urine (12 studies) and fecal samples (7 studies). Taken together, a total of 829 unique metabolites, including non-phenolic compounds, were identified in clinical samples (**Figure 4A**). Similar to the observations reported in food composition, the

categories of metabolites that comprised the greatest number of unique compounds detected in clinical samples included flavonoids and hydroxycinnamic acids (**Figure 4B**), and the categories with the greatest frequency of detection were flavonoids, hydroxycinnamic acids and benzoic acids (**Figure 4C**). However, the most frequently detected unique metabolites included phenylacetic acid, gallic acid and hippuric acid (**Figure 4E**).

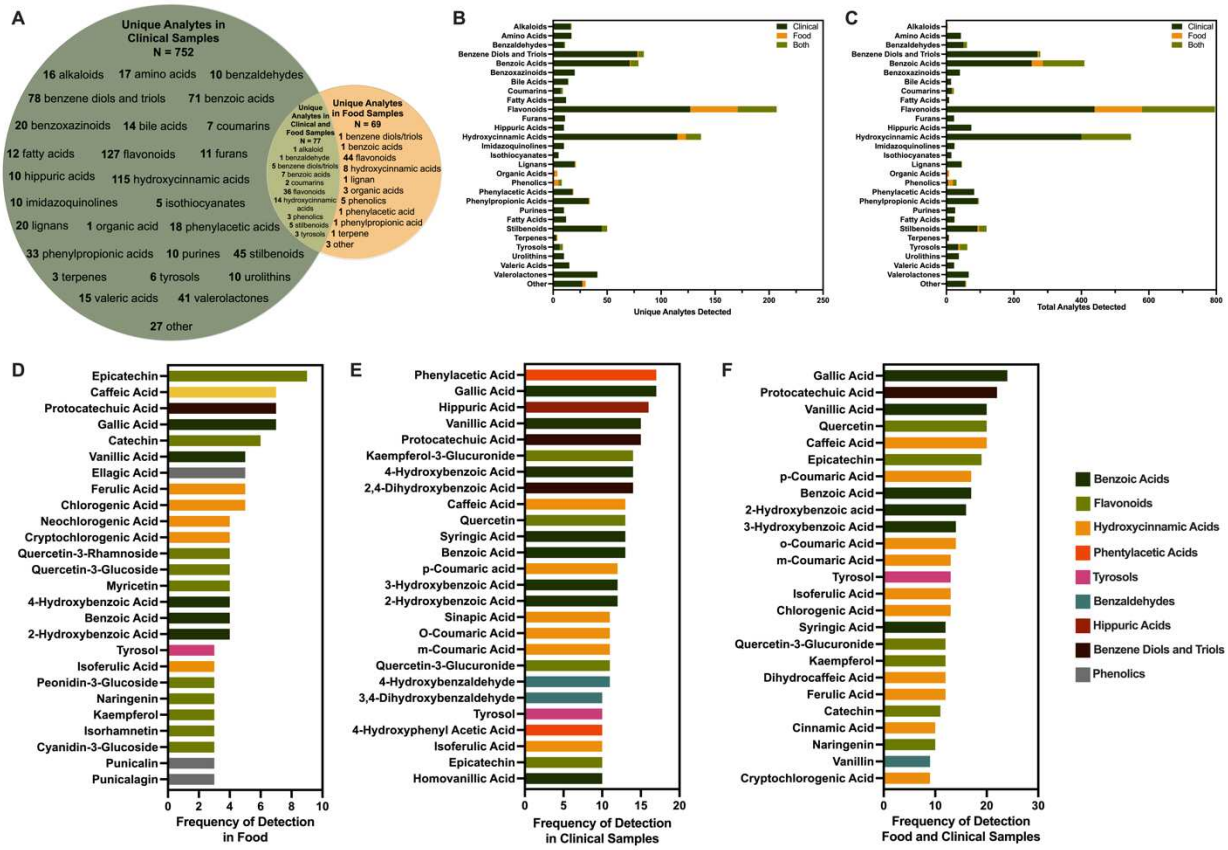

Figure 4 Comparison of metabolite identification in food interventions versus clinical samples. (**A**) A greater number of unique analytes were identified in clinical samples (752) than in food samples (69). 72 unique analytes were identified in both. (**B**) Classification of unique analytes by chemical structure demonstrated the emphasis on flavonoids, hydroxycinnamic acids, benzene diols/triol and benzoic acids across both clinical and food samples. (**C**) Flavonoids, hydroxycinnamic acids, benzoic acids and benzene diols/triols were the most frequently detected analytes. Overall frequency of detection of analytes in (**D**) food, (**E**) clinical samples and (**F**)

both demonstrates prioritization of flavonoids, benzoic acids, benzene diols/triols and hydroxycinnamic acids in targeted approaches.

Untargeted Analyses for Clinical Samples

Of the 42 papers included in this review, only five used untargeted analyses. In each case, these techniques were applied only to clinical samples (e.g., plasma, urine). Two of these studied combined untargeted approaches with targeted, allowing for quantitation of specific metabolites.

Untargeted analyses are most notably distinct from targeted analyses based on the breadth of data collected and the ability to identify metabolic fingerprints based on clinical interventions. For example, individuals with metabolic syndrome who consumed 30 g nuts/day were found to have a distinctly different plasma metabolome than the control group, allowing for identification of metabolites that can serve as biomarkers of dietary exposure. Furthermore, these data were used to generate novel hypotheses related to links between nut intake and cardiometabolic risk based on correlations between observed plasma metabolites and clinical parameters of metabolic syndrome [61]. In contrast to the high-resolution analyses applied to the clinical samples, a database was used to infer the polyphenol content of the food intervention. This highlights a significant gap in the detailed analysis of foods in clinical trials, emphasizing the need for more thorough characterization of dietary interventions to match the level of detail provided for clinical markers.

Another example of untargeted approaches in a clinical trial is the evaluation of wheat aleurone supplemented foods on cardiovascular disease [40]. The randomized, double-blind, placebo-controlled trial employed extensive analytical tools to characterize the metabolomic profiles of participants' urine and plasma. In urine, over 16,000 metabolites were detected with 656 found to be discriminant between treatment groups. Likewise, in plasma, over 8,000

metabolites were detected with only 18 found to be discriminant between dietary treatment groups. The authors annotated metabolites into families based on chemical similarities and performed correlations among anthropometric and clinical parameters, fecal microbial taxa, and urinary and blood metabolites. The primary goal of that study was to investigate the effects of aleurone supplementation on plasma homocysteine levels in individuals with overweight or obesity: however, no statistical differences were observed. While previous studies have reported statistically significant reductions in plasma homocysteine, the authors hypothesized that the lack of effect could be due to their use of a less purified form of aleurone. Notably the study did not include metabolomic analysis of the food itself. The only compositional data provided was a table listing the caloric (kcal) and macronutrient profile (proteins, carbohydrates, fats, and dietary fiber in grams) and aleurone extract content (grams). This omission makes it challenging to draw a definitive conclusion about the potential differences, as the lack of input analysis- in this case, wheat aleurone- limits the interpretability of the findings.

While untargeted analyses were not applied to the dietary interventions in any of the five studies using untargeted analyses on clinical samples, one study did acknowledge and control for variation in phenolic composition in their intervention using a targeted approach [88,89]. This study aimed to investigate the effects of apple intake on the gut microbiome and co-metabolic processing of polyphenols, tyrosine and tryptophan. Control was exercised over this intervention by not only supplying participants with a specified apple cultivar procured from the same orchard, but they also used a targeted approach to ensure that the phenolic composition of the apples did not vary between batches or over the course of storage [89]. The resulting identification of 61 urinary metabolites and nine plasma metabolites reflective of apple intake

has greater translatability for other studies, as future interventions can be compared to their intervention data alongside comparison of clinical findings.

Identifying a Role for Foodomics in Precision Nutrition Research

Precision nutrition research relies on identifying and acknowledging unique characteristics of individuals which may influence responsiveness to nutritional interventions. This research area has been supported by the implementation of advanced analytical technologies including characterization of the human metabolome in response to dietary bioactive compound intake. Similarly, recent advances in food science and analytical chemistry have led to the establishment of the field of foodomics, revealing complex and nuanced differences in the chemical composition of foods.

The objective of this review was to characterize existing practices in characterization of food composition in clinical nutrition intervention trials investigating the impact of polyphenols on the gut microbiome. Our findings revealed that despite widespread use of untargeted analyses in clinical samples (e.g., characterization of human metabolome), similar comprehensive analyses were not applied to intervention foods. Instead, analyses included targeted analytical methods, which focus on pre-selected analytes, and nonspecific methods, which focus on concentrations of broad classes of compounds. These overarching classes of analyses can provide advantages over the use of databases, and targeted analyses provide even greater advantages over nonspecific methods for food characterization by overcoming biological interferences; however, neither allow for broad characterization of the food metabolome and thus represent lower resolution analyses than those applied to clinical samples. The addition of more comprehensive characterization of food interventions in clinical studies has the potential to support systems-

level insights into the relationship between food and health, wherein increasing the level of precision applied to ventures like precision nutrition. Though our analysis encompasses only a subset of clinical research (i.e., studies within a specified timeframe focused on the influence of polyphenols on the gut microbiome), the complete absence of untargeted analyses in characterization of food interventions suggests that implementation of these methods is likely similarly limited across the field of clinical nutrition research. Further systematic reviews are needed to test this hypothesis and identify gaps in the knowledge.

Public Policy Implications

The implementation of comprehensive characterization of food composition into clinical research has the potential to support ongoing advancement in public policy related to nutrition-focused approaches to support human health. While policies such as the 2020-2025 Dietary Guidelines for Americans advocate for a healthy eating plan for the general population and dietary reference intakes guide adequate nutrient intake for life, growth and development across the lifespan, these recommendations exist only for macro- and micronutrient intake and thus do not consider the molecular complexity of foods or the potential for other non-nutritive components to influence human health and wellness.

In response, there is an emerging consensus that dietary bioactive components should be considered in food-based approached for dietary recommendations, spearheaded by regulatory agencies and professional societies. In recent years, the Food and Drug Administration and the European Food Safety Authority have approved qualified health claims related to the intake of overarching classes of phytochemicals including flavan-3-ols [90,91] and phytosterols [92,93] to support cardiovascular health for at-risk populations. Similarly, the Academy of Nutrition and

Dietetics recently developed the first US-based guideline for dietary intake of a bioactive component. Specifically, recommendations for flavan-3-ol intake (including dietary approaches for obtaining recommended intakes) for the general population were provided based on aggregate evidence of risk reduction across multiple cardiometabolic biomarkers and endpoints [94].

These developments demonstrate the value of research that includes compositional characterization and underscores the continued need for standardized, comprehensive and reproducible analytical approaches in nutrition research. Consistent methodology for the characterization of bioactive composition in food has the potential to support improved translatability of outcomes into public policy and recommendations.

Developing Framework for the Implementation of Foodomics in Clinical Research

To support the implementation of foodomics into routine clinical application, we propose a structured framework that integrates methodological rigor, interdisciplinary expertise and standardized practices.

Integration of Foodomics and Clinical Outcomes

Numerous studies have established that untargeted metabolomics can reveal distinct metabolite profiles within foods, and others have identified distinct metabolite signatures in clinical samples verifying intake of these foods even against the background of complex habitual diets [95]. This has been demonstrated across an array of polyphenol-rich foods like cocoa, coffee and fruits, in studies where participants either ate their typical diet and reported intake via food frequency questionnaires [96,97] or consumed a novel intervention food on a single test day

amidst a typical diet [98]. In these and other studies, distinct gut microbial metabolites and minor phenolic derivatives are used to reflect the consumption of specific polyphenol-rich foods (e.g., methylxanthines from cocoa, ferulic acid derivatives from coffee). Notably, this approach has been used in clinical nutrition research to identify food-specific metabolites in human plasma in association with improvements in cardiovascular health indicators [99], revealing a path forward for the integration of complex datasets from intervention foods, biological samples collected from participants and clinical indicators of health and disease.

With more widespread use in clinical settings, untargeted metabolomics of intervention foods may provide critical information for understanding the effective dose of bioactive food compounds. Because untargeted approaches monitor a wide array of metabolites, observations of relationships between metabolites in foods versus biological samples (e.g., stool, plasma) have the potential to elucidate complex mechanisms related to metabolism and biological effects which may be oversimplified without the context of the food metabolome delivered to a participant via an intervention food. Though untargeted approaches are only semi-quantitative, they offer valuable complementary information to more quantitative, targeted approaches, which has the potential to maximize insights into dietary metabolite dynamics and improve the precision of precision nutrition approaches [4].

Interdisciplinary Collaboration for Development of Standardized Methods

There are several factors that likely contribute to the lack of inclusion of foodomics in clinical research. First, the cost associated with such analyses is high as mass spectrometry and chromatography systems are expensive to acquire and maintain. Furthermore, the generation of

foodomics data requires expertise that may be outside the area of expertise of clinical study leaders. Finally, the large and complex datasets generated by untargeted omics presents challenges in terms of data interpretation. Effective analysis of the datasets requires a robust foundation in statistics to prevent false discoveries and draw meaningful conclusions. Ultimately, one or more of these and other factors may be barriers to inclusion of such research approaches but can be mitigated through collaboration and inclusion in grant proposals.

As previously mentioned, there is no single analytical method that can characterize all of the compounds in a food. Even a specific class of compounds such as polyphenols may require multiple analytical methods to capture the full diversity within a food. For example, proanthocyanidins, also referred to as condensed tannins, are more challenging to characterize, compared to smaller polyphenols due to their high degree of polymerization and structural complexity [100]. Outside of polyphenols, other bioactive compounds such as phytosterols and carotenoids often require distinct analytical approaches due to their hydrophobic properties. Nevertheless, multiple studies have demonstrated that generalized untargeted methods can detect an extensive range of metabolites [101–103]. While targeted methods have the advantage of more precise quantitation, untargeted analyses enable a holistic characterization of small molecules in food, allowing for robust comparison between interventions across different trials and the identification of potential novel pathways for biological function of bioactive compounds such as polyphenols. These analyses should not be considered either/or, but complementary, especially in consideration of the structural diversity of polyphenols and the limited availability of analytical standards for targeted analysis.

Perhaps one of the largest barriers in the utility of data from untargeted analysis is the lack of standardization for sample extraction, data acquisition, and data processing across laboratory

which introduces analytical bias and inhibits comparability of findings across different studies. To advance the incorporation of omics analysis and data in clinical nutrition research, there is a critical need for the development and implementation of standardized analytical methods and reagents for data acquisition, approaches for data processing, and a unified database to host the data. This would not only enhance the reliability of study outcomes, but also enable large-scale discoveries made by mining data across studies, ultimately driving the goals of precision nutrition research. The Periodic Table of Food Initiative (PTFI; https://foodperiodictable.org) is an example of such an effort [5]. Briefly, the PTFI has developed and validated standardized omics methods including metabolomics, lipidomics, total fatty acid analysis (FAMES) and ionomics for the analysis of food which are being implemented in laboratories around the globe. A major goal of the PTFI is to improve reproducibility and validity of foodomics. Data generated from these laboratories is housed within a centralized and accessible database to enable comparison of data and drive discovery. Ultimately, such initiatives highlight the utility of fostering interdisciplinary collaboration to ensure that researchers, clinicians, and data scientists can effectively generate, interpret and apply foodomics data to support the advancement of precision nutrition approaches to improve human health.

Conclusions

To advance the field of precision nutrition, researchers and stakeholders must prioritize investment in the foodomics research. Untargeted metabolomics can complement targeted approaches by broadening understanding of dietary polyphenols and their complex interactions with the gut microbiome and other aspects of human health. It represents a critical tool that can bridge the gap with precision nutrition, reshaping our understanding of the relationship between

diet and health and ultimately improving global health outcomes. With its high dimensional data, more refined questions can be answered- such as not just whether “an apple a day keeps the doctor away”, but which apple, and why.

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CHAPTER 2: THE EFFECT OF SOURDOUH STARTER CULTURE ON THE CHEMICAL COMPOSITION OF SOURDOUGH BREAD.

Abstract

Bread is a global staple, and consumer demand is shifting toward products with targeted quality and health attributes. Emerging evidence links sourdough microbiome composition to bread quality, highlighting fermentation as a technology to enhance nutritional functionality. Here, we applied standardized Periodic Table of Food Initiative (PTFI) workflows to examine relationships between 20 divergent sourdough microbiomes and the chemical composition of dough and bread. Twenty sourdough starters and three commercial baker's yeasts were used to prepare triplicate dough and bread samples. Metabolomics was performed using reverse-phase LC on an Agilent Zorbax SB-Aq column coupled to a Waters Synapt G2 Q-TOF, acquiring MS1 data and data-dependent MS2 for pooled quality controls. 16S rRNA sequencing revealed variation among starter microbiomes. Diverse phenolics, terpenes, and alkaloids were detected, with yeast controls clearly distinct from sourdough products. Compound annotation using the PTFI consensus library enabled correlation of specific microbiome compositions with metabolites with known anti-inflammatory, antibacterial, and antioxidant properties. These findings suggest that microbial composition may influence the stability and nutritional quality of bread.

Associated authors Eva Keohane, Ashley Ostrom, Josephine Wee, Rachel Jones, Charlene Van Buiten, Jessica Prenni.

Introduction

Sourdough bread is a traditionally fermented food made by naturally leavening dough with a starter culture which consists of wilds yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB). These microorganisms work synergistically to produce CO_2, which aerates and leavens the dough. Research has shown that the specific composition of these starter cultures can significantly influence various bread attributes including flavor, loaf volume, and crumb firmness, among other properties [18][19][20]. Furthermore, these microbial communities are responsible for the production of bioactive compounds that can enhance shelf-life stability and offer potential health benefits for consumers [21], [22].

Despite prior research on the composition of sourdough bread, a knowledge gap remains regarding how specific starter cultures influence the functional and nutritional quality of the resulting sourdough bread. Previous studies typically focused on aroma compounds, used targeted methods to quantify specific compounds, or consider all sourdough starters to be the same [23], [24]. While some studies have performed a more comprehensive analysis using nontargeted metabolomics, these only examined isolated yeast or bacteria and their effects on bread [21]. Previous work by Landis *et al.* demonstrated significant diversity of bacteria and yeast taxa within 500 different sourdough starter cultures. The sources of these cultures ranged from large scale bakeries to at home bakers across the world [25]. They found that geographic location does not determine total microbiome composition but may influence the presence of specific taxa. Furthermore, they evaluated a subset of the 500-starter cultures to characterize the influence of acetic acid bacteria on sensory properties such as volatile organic compounds. They detected 123 volatile organic compounds (VOCs) using gas chromatography mass spectrometry (GC-MS) in sourdough doughs and observed a strong correlation between the microbial

composition correlation and the VOC profiles. However, it is unknown whether compositional diversity correlates with function in the context of bread quality and human health.

The objective of this study is to understand the relationship between the metabolite composition of bread generated by the starter culture microbiome and nutritional and functional bread quality. We focus our analysis on the small molecule composition of both dough and bread as a mechanism to evaluate the relationship between the microbial function of the starter culture and the chemical composition of the dough and bread. Understanding the microbial impact of the starter culture will enable a deeper understanding of how starter culture microbial ecology affects small molecules in bread, enabling the targeted enhancement of nutritional functionality for human health and shelf-life stability to reduce food waste.

This study represents a unique multi-omics evaluation of multiple starter culture microbiomes and their impact on the chemical composition of dough and bread. As expected, our findings reveal distinct chemical differences between yeast-leaved and sourdough breads. Notably, the relationship between the microbial composition of starter cultures and the chemical composition of bread is complex and cannot be explained solely by microbial membership. These findings highlight the need for further research to fully understand the extent and mechanisms underlying this influence. We also examined functional redundancy within microbial communities and its correlation with the functional attributes. In addition, we conducted a cell assay to assess the inflammatory effect of bread extract.

This work carries important implications for both food systems and human health. Certain small molecules produced by these microorganisms can enhance food preservation and extend shelf-life[26]. Additionally, it addresses human health concerns, given the rising

prevalence of chronic gastrointestinal conditions, which has spurred increased interest in foods that promote digestive health and reduce inflammation[27]. Together these findings demonstrate the value of multi-omics approaches for bridging microbial ecology, food chemistry and human health, providing a foundation for future studies aimed at improved food quality and health outcomes.

Materials and Methods

Starter Cultures and Bread Baking

Twenty sourdough starter cultures were obtained from the science-based global sourdough project, selected to represent unique microbial communities based on prior assessments of beta and alpha diversity [25]. The starters were stored at -80ºC (Tufts University Medford, MA) before being shipped to Colorado State University, where they were reactivated. For propagation, starters were feed with all-purpose flour (King Arthur Baking Company, Inc.) and autoclaved water. Initial activation involved combining 5 g of each stater with 10 g of a 1:1 mixture of autoclaved deionized water and flour, followed by further feeding steps to reach a total starter weight of 375 g.

Active sourdough starers were used to create 1 kg of dough (65% hydration) by combining 167 g sourdough starter, 335 g King Arthur all-purpose flour, 188 g autoclaved dH2O and 10 g non-iodized kosher salt. Three brands of *S. cerevisiae* were used as control doughs were prepared using three commercial brands of *S. cerevisiae,* matched for ingredient composition and hydration level containing 415 g King Arthur all-purpose flour, 265 g autoclaved dH_2O, 10 g non-iodized kosher salt, and 6 g active dry yeast (Bob's Redmill, Fleischmann's and Redstar).

Control doughs were prepared using three commercial brands of *S. cerevisiae,* matched for ingredient composition and hydration level. Yeasts were pre-proofed in water, and all doughs were mixed and kneaded in sanitized mixers. Sourdoughs and yeast doughs fermented at 25 °C and 30% for 24 and 2 hrs, respectively. Following fermentation, samples from each dough were collected and stored at -80 °C. Then loaves were shaped and baked in preheated ovens at 204°C until reaching an internal temperature of 93 °C. After cooling, breads were cut and stored at -80 °C.

Metabolomics

Samples were collected after dough fermentation and post-baking, then stored at -80°C. Samples were processed and analyzed using the standardized metabolomics method developed by the Periodic Table of Food Initiative (PTFI). Briefly, frozen samples were lyophilized to dryness (~ 72 hrs) using a Labcono Lyophilizer (Kansas City, MO), then homogenized using a coffee grinder [28]. Homogenous, powdered samples were sieved onto a sheet of aluminum foil and subsequently transferred to a plastic storage bag [29]. Once homogenized, samples were stored at -80 °C prior to aliquoting of 55 ± 5 mg of sample. Samples were stored at -80 °C and shipped on dry ice to the analysis laboratory for the completion of small molecule extraction and LC-MS1 data acquisition with the PTFI standardized metabolomics protocol (Version 1.2.20240805).

Reverse-phase metabolomics extraction was standardized following a previously published method for characterizing small molecules in food [30]. Captiva EMR-Lipid Cartridges (Agilent Technologies, Santa Clara, CA) were first conditioned using 120 µL of 80%

Methanol: Water (MeOH:H_2O) and 480 µL of crash solvent (1% of formic acid with 5:1 Acetonitrile: Water). A positive pressure manifold was used to dispense 1 drop of solvent every 3-5 s. Captiva cartridges were left under nitrogen for an additional 10 minutes to ensure complete dryness prior to loading samples. Meanwhile, lyophilized samples and additional samples of an orange carrot to serve as a system suitability test were allowed to reach room temperature prior to reconstitution in 12 µL of 80% MeOH:H_2O for every 1 mg of food. Blank samples were also created using 600 µL of 80% MeOH:H_2O. Samples were vortexed for 10 min at room temperature and then centrifuged at 14000 RCF for 10 min at room temperature. Cartridges were loaded with 480 µL of crash solvent, 120 µL of supernatant from each sample, and 10 µL of a synthetic mix of 34 compounds non-endogenous to food (Analyticon Discovery, Potsdam, Germany).

For solid phase extraction (SPE), nitrogen flow was set to dispense 1 drop of sample every 3-5 seconds. Effluent from each sample was collected in autosampler vials below each Captiva Column. Once the sample fully flowed through the cartridge, the autosampler samples were capped and centrifuged for 10 min at 3500 RCF before drying with nitrogen. Samples were reconstituted in 160 mL of 80% MeOH:H_2O, vortexed for 10 minutes at room temperature and centrifuged at 3500 RCF for 10 minutes at room temperature prior to storage at -80 ºC until LC-MS analysis.

Reverse-phase metabolomics data acquisition was standardized following a previously published method for characterizing small molecules in food [30]. Nontargeted metabolomics data was collected using a Waters Acquity BSM coupled to a Waters Xevo G2-XS qTOF mass spectrometer. LC separations were completed using a reverse phase Agilent Zorbax SB-Aq RRHD column (2.1 mm × 100 mm × 1.8 µm particle size) with an Agilent in-line filter (0.3 µm,

2 mm I.D.). Samples (2 µL injection) were separated over a 15-minute linear gradient of mobile phase A consisting of 100% Water with 0.1% Formic acid (FA) and mobile phase B consisting of 100% Acetonitrile with 0.1% FA. The LC-gradient increased mobile phase B from 1.8% to 90% over 11 minutes following a 1-minute hold of starting conditions. 90% B was held for 1.8 minutes before re-equilibrating the column to starting conditions.

Samples were analyzed in both positive and negative ESI mode using separate injections at a resolution of 15,000 in centroid mode. Throughout instrument acquisition of samples, a reference spray of Leucine Enkephalin was measured periodically. Mass data was auto-corrected relative to this reference. For LC-MS1 data acquisition, samples were analyzed in a randomized order with pooled quality control (QC) samples analyzed every 7 injections and a system suitability reference food of carrot analyzed every 20 injections. During acquisition, data quality of samples was monitored in Skyline (v22.2). Additional instrument-specific settings are presented in Table 1.

Table 1. Waters Xevo G2-XS Mass Spectrometer Settings

Parameter	Positive Ionization Mode	Negative Ionization Mode
MS TOF		
m/z range	50-1200	50-1200
Scan time (sec)	0.3	0.3
StepWave		
StepWave RF (V)	300	300
StepWave Ion Guide (V)	350	350
ESI Source		
Source Temperature (C)	150	150
Cone gas (L/hr)	50	50
Desolvation Temperature (C)	450	450
Desolvation gas (L/hr)	1000	1000

Capillary Voltage (V)	700	1700
Sampling Cone (V)	40	40
Source Offset (V)	80	80
Lockspray settings		
LockSpray Capillary (V)	2000	2000
Reference Mass (Leucine Enkephalin)	556.2771	554.2615

Data processing and annotation was performed using the PTFI V2 Data Processing Pipeline as previously described (Tech Note). Briefly, centroided, mass-calibrated raw data files were first converted to mzML format using MSConvert (Version 3.0.23144-0ae01f1)[31][32]. The instrument-specific parameters were used to optimize feature finding for data from each laboratory using XCMS (Version 3.12)[33][34]. XCMS data was converted from retention time to retention index (RI) using a regression assembled from IRTS signals across each injection. For samples where multiple features match an IRTS compound, the analyte with the highest intensity is selected for regression construction. To circumvent the false assignment of one or a small set of IRTS candidates interfering with regression fit, a Tukey M-estimator is used to apply a robust regression to the data without assuming a normal distribution [35][36]. The resulting 2^{nd}-order polynomial fit scales the raw retention time values to RI.

MS1 features at particular RIs (feature-RIs) are annotated as follows. (1) Masses are searched against the monoisotopic masses of entries in the PTFI Global Consensus Library (GCL). The GCL contains both named compounds (based on the analysis of authentic standards) and unknowns annotated with molecular formula. Masses are searched using the following adducts in positive mode: $[M+H]^+$, $[M+H-H_2O]^+$, $[M+Na]^+$, $[M+NH_4]^+$, $[M+K]^+$ and negative mode: $[M-H]^-$, $[M-H-CHOO]^-$, $[M-H-H_2O]^-$, $[M-Cl]^-$, $[M+Na-2H]^-$, $[M+K-2H]^-$; respectively. (2) Masses that match within 15 ppm and RI difference < 10 of a GCL entry are

tentatively assigned annotation. (3) Annotations are further validated by comparing the isotopic distribution of the experimental compound to that of the tentative annotation. Data is normalized to the intensity of the median IRTS within each injection.

Amplicon 16S and ITS rRNA sequencing

Samples were collected and shipped to Wright Labs, LLC. Nucleic acid extractions were performed on approximately 0.25 g of each sample using a Qiagen DNeasy PowerSoilDNA Isolation kit following the manufacturer's instructions (Qiagen, Frederick, MD). The lysing step was performed using the Disruptor Genie cell disruptor (Scientific Industries). Finally, the genomic DNA was eluted in 50 µl of 10 mM Tris. Subsequent quantification was performed using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA) with the double-stranded DNA high-sensitivity assay.

llumina iTag Polymerase Chain Reactions (PCR) were performed based on the Earth Microbiome Project's ITS amplification protocol. The volume of each reaction was 25 µL and contained (final concentrations) 1X PCR buffer, 0.8 mM dNTP's, 0.625 U Ex TaqDNA Polymerase (Takara), 2 µL of template DNA diluted to between 1:5 and 1:100 DNA: Sterile Water to optimize the reaction due to high extract concentrations, and barcoded primers. For ITS 0.2 µM ITS1F forward primer, 0.2 µM ITS2R reverse barcoded primers were used, and for 16S 0.2 µM 515F forward primer and 0.2 µM 806R reverse barcoded primers were used. Amplification was carried out on a T100 Thermal Cycler (Bio-Rad, Hercules, CA) using the following cycling conditions: 94 °C for 1 min; then 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 68 °C for 30 s; final extension was at 68 °C for 7 min; then held at 4 °C until further

processing. PCR products were visualized on a 2% agarose E-Gel within gel ethidium bromide (Thermo Fisher Scientific).

Purified libraries were pooled and sequenced on the Illumina NextSeq platform using the 150 bp dual-ended chemistry at the University of California Davis Genome Center. Sequencing data for each sample was processed with the R package DADA2 v1.30.0 pipeline following standard protocol for bacterial 16S rRNA V4 gene region (515f/806r) and the fungal ITS gene region (ITS1f/ITS2)[37]. Low-quality sequence reads were filtered, low-quality bases were trimmed, error rates were calculated, and amplicon sequence variants (ASVs) were inferred from the remaining sequence reads. Paired end reads ASVs were merged into contigs and ASVs with <250 bp or >253 bp were removed for bacterial 16S reads. ITS reads were not selected based on length due to biological variation of fungal ITS gene region. The remaining ASVs were assigned taxonomy using the Silva database v132 for bacteria [38] and the UNITE v8.3 database for fungi [39]. ASVs assigned to mitochondria, chloroplasts, and reads unassigned at the phylum or class levels were filtered out from the bacterial dataset. ASV tables were rarefied to 1260 bacterial reads per sample and 4000 fungal reads for downstream analysis.

Functional predictions were generated using the Quantitative Insights into Microbial Ecology version 2 (QIIME2) pipeline[40]and the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)[41]. Paired-end 16S sequences were imported into QIIME2 v2024.8, where they were quality filtered to remove low-quality reads. Sequences were then denoised and dereplicated, with a trim length of 120 bp. Taxonomy was assigned using a Naive Bayes classifier trained on the Greengenes database, and sequences identified as mitochondria or chloroplast were removed. The outputs from QIIME2 were used to run PICRUSt to predict enzyme commission (EC) numbers, KEGG orthologs (KOs), and metabolic

pathways, which were subsequently exported for downstream analyses. The EC numbers predicted by PICRUSt were used to categorize enzymes into different biological function groups for each sourdough sample.

Radical Scavenging Capacity

Bread samples were dissolved in 0.2 N HCl at a rate of 20 mg/mL with pepsin added at a final concentration of 0.3 mg/mL and incubated at 37°C under agitation for 1 h. The pH of each sample was then increased to 6.4 with 2 N NaOH and trypsin was added at a rate of 0.3 mg/mL per final volume. Samples were then incubated under agitation for 2 h. After incubation, samples were boiled for 5 min and frozen at -80°C before lyophilization. Lyophilized bread digesta was dissolved in 80% ethanol at a rate of 1 g bread per 5 mL and sonicated for 15 min before centrifugation. The supernatant was reserved for analysis of radical scavenging capacity by the 2-2'-azino-bis(3-ethylbensothiazoline-6-sulfonic acid (ABTS) assay according to previously established protocol[42]. Radical scavenging capacity is expressed in Trolox equivalents.

Statistical Analysis

Raw peak intensities obtained from the PTFI data analysis pipeline were filtered to include only those with an m/z diff of 10 ppm. Missing values were imputed by replacing them with one-fifth of the minimum positive value for each individual feature with 10% random values added for noise. Scaling by z-scores and raw peak intensities were used for data visualization, depending on the plot. Partial Least Squares Discriminant Analysis (PLS-DA) was

then conducted to identify patterns in the dataset. The quality of the PLS-DA models was evaluated using R^2 and Q^2 metrics to confirm the model's robustness against overfitting. ANOVA analysis was done on the dataset to compare between the four groups and identify the top 50 compounds to form a heatmap. A two-sample t-test was performed with a significant threshold of $p < 0.05$, adjusted for multiple comparisons using false discovery rate (FDR) correction. Sample t-tests and Welch's t-tests were chosen for their suitability in analyzing normally distributed data, with the former applied to equal sample sizes and the latter accommodating unequal sample sizes, while both allow assessment of significance between groups. Amplicon Sequence Variants (ASVs) from 16S rRNA sequencing and PICRUST enzyme predictions were imported into R (Version 2024.12.1+563), where hierarchical clustering analysis (HCA) was performed to classify samples into distinct sourdough bread groups. These groups were subsequently used as class labels in R for PLS-DA model building.

Results and discussion

Chemical diversity of yeast and sourdough doughs and breads.

Overall, 1,995 features were detected across all samples. Of these, 211 compounds (10.6%) were annotated. The annotated compounds were categorized by chemical class as: 30.0% lipid and lipid-like-molecules, 29.1% phenylpropanoids and polyketides, 16.3% organic acids and derivates, 7.4% benzenoids, 6.4% organic oxygen compounds, 4.9% organoheterocyclic compounds, 2.5% nucleosides, nucleotides, and analogues, 1.5% lignans, neolignans and related compounds, 1.0% alkaloids and derivatives, and 1.0% organic nitrogen compounds. The compound class of phenylpropanoids and polyketides is of particular interest as

it includes polyphenols which are well recognized for their bioactivity and studies have revealed health benefits including anti-inflammatory, anti-carcinogenic, and cardioprotective effects [43], [44][45]. Antioxidant activity is primarily due to their ability to scavenge free radicals, thereby reducing oxidative stress in biological systems [46], [47]. The complexity of human metabolism and interaction with the gut microbiome may influence the bioavailability and activity of these compounds, highlighting the need for further research to elucidate their functional roles and health implications especially within diverse food matrices [48], [49].

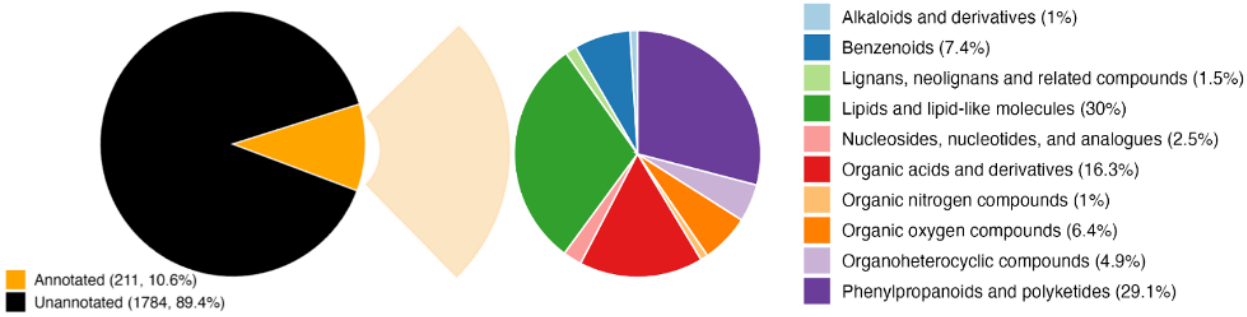

Figure 1. (Left) Total detected compounds across all bread and dough samples using non-targeted metabolomics. (Right) Breakdown of annotated compounds by chemical class.

Partial Least Squares Discriminant Analysis (PLS-DA) revealed the overall variation in the chemical composition between the doughs and breads (Figure 2). Each point in the plot represents a sample replicate, with colors distinguishing the groups: sourdough bread, sourdough dough, yeast bread, and yeast dough. The distance between points reflects the similarity or dissimilarity of the samples.

As expected, a clear separation is observed between bread and dough samples, likely reflecting the breakdown and evolution of compounds during the baking process. This

phenomenon aligns with previous research demonstrating the impact of food processing, such as baking, on the chemical profiles of food [50]. Notably, separation is also observed between the sourdough and yeast samples, highlighting the influence of leavening agent on the chemical composition, reflecting the fermentation process which involves the microbial metabolism of macromolecules into small metabolites [51]. This separation is driven by 630 compounds with an average variable importance score (VIP) > 1, including both annotated and unknown metabolites.

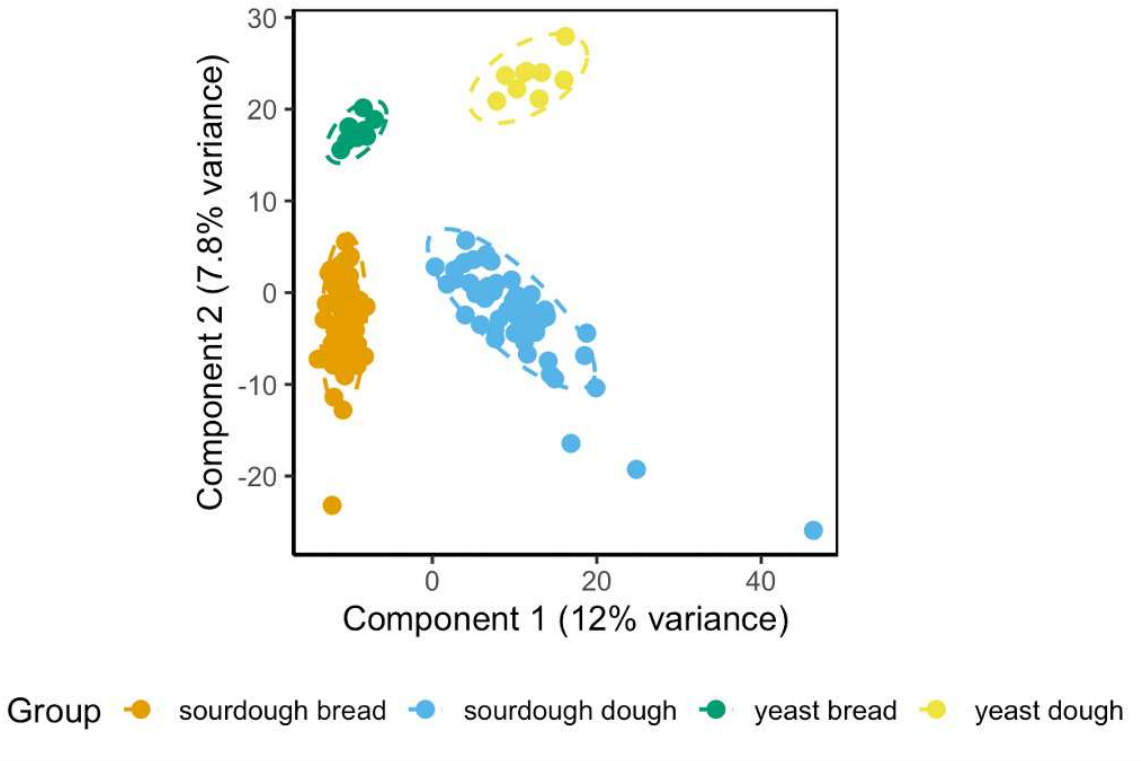

Figure 2. Scores plot from Partial Least Squares Discriminate Analysis where each point represents an individual replicate of sourdough and yeast, bread, and dough. Components 1 and 2 describe 12% and 7.8% of the variation in the dataset, respectively. Cross validation accuracy of the model was 0.993, 0.935, 0.949, and 0.949 for sourdough bread, yeast bread, sourdough dough, and yeast dough, respectively.

Variation in chemical composition between dough and bread.

The heatmap of the 50 most significant entities identified through ANOVA reveals a clear pattern that distinguishes doughs and breads (Figure 3). Among the annotated compounds, linoleic acid is notable as an essential polyunsaturated fatty acid that is obtained from dietary sources[52]. Similarly, α-linolenic acid and ethyl linolenate exhibit higher relative abundances in dough (Figure 4) and have a VIP score > 1 contributing to the separation between breads and dough (Figure 2). Linoleic acid, α-linolenic acid, and ethyl linolenate are all polyunsaturated fatty acids which are highly susceptible to thermal degradation[53]. During baking, elevated temperatures and oxygen exposure can promote lipid oxidation, leading to the formation of volatile compounds resulting from fatty acid breakdown[54],[55]. This supports our data, where a significant decrease of these essential fatty acids is observed in the bread compared to the dough (Figure 4). Notably, we observed greater variation in the abundance of these compounds in dough compared to bread (Figure 4). Previous research has shown free lipid extracts contain higher levels of PUFAs in flour and sourdough than in the baked bread, whereas bread exhibits increased levels of bound and starch associated lipids after baking [56]. In relation to our data, this suggests that some PUFA may be modified during baking resulting in lower relative abundance in the final breads.

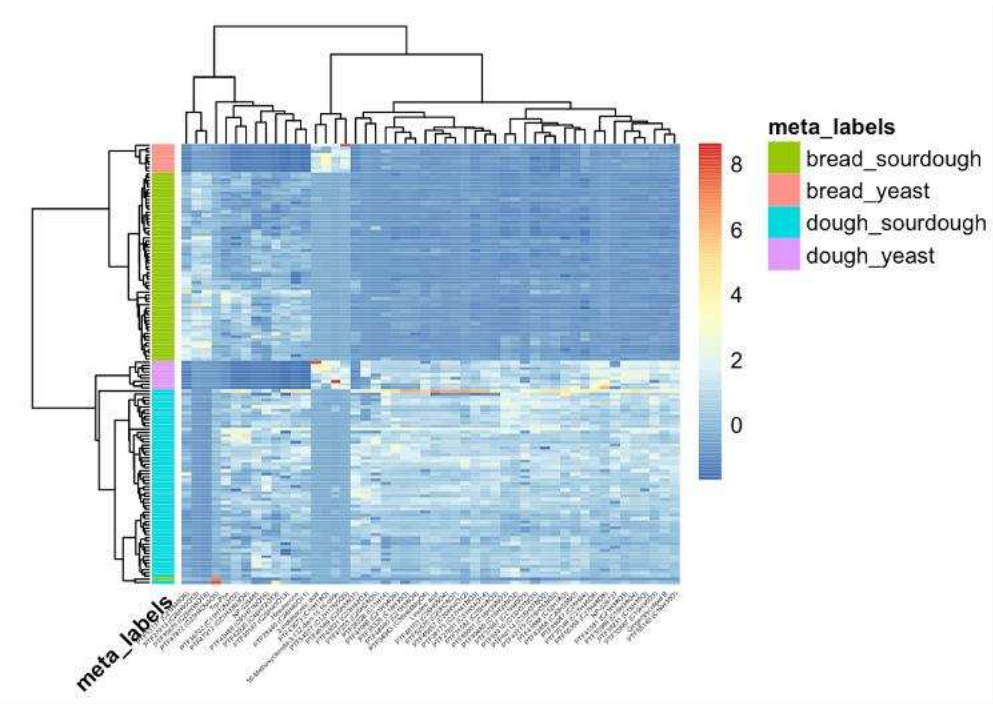

Figure 3. Heatmap illustrating compounds top 50 features from ANOVA. The color intensity represents relative abundance across samples, highlighting features with differential abundances between groups, sourdough bread, yeast bread, sourdough dough, and yeast dough. Along the x-axis depicts annotated and unannotated entities. Unannotated entities are notated with PTF. Y-axis is grouped through HCA clustering of the top 50 features.

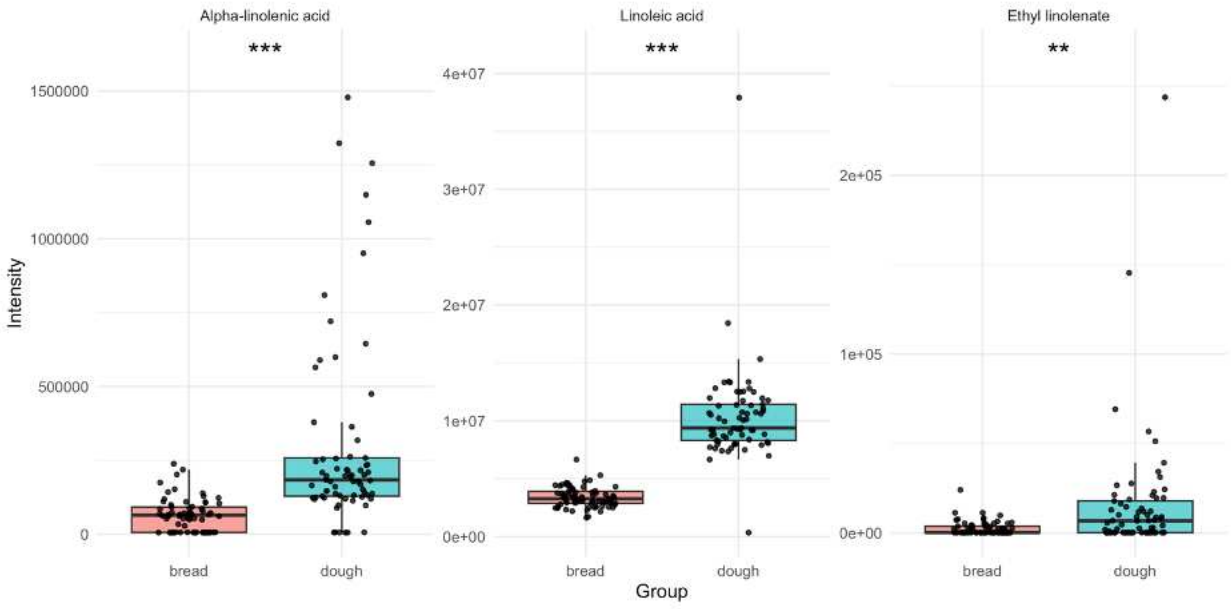

Figure 4. Box plots of 3 of the 673 detect entities, alpha-linolenic acid, linoleic acid, and ethyl linolenate, with a VIP score >1 from the PLSDA that contribute separation of doughs and breads. Sample t-test was used to determine significance between bread and dough groups (BH adjusted p-value, * < 0.05, *** <.001).

Variation in chemical composition between sourdough and yeast bread.

Based on the PLS-DA separation, clear differences were observed between sourdough and yeast breads (Figure 2). Specifically, 569 entities with a VIP score >1 contributed to this separation. Several annotated compounds with a VIP score >1- including 3-phenyllatic acid, azelaic acid, caffeic acid, dihydrocaffeic acid, folic acid, and Kaempferol- played a key role in distinguishing yeast and sourdough (Figure 5). Phenyllactic acid, for example, was more abundant in sourdough samples, likely due to fermentation driven production of bioactive compounds[57]-[58]. Previous work suggests a possible pathway of the formation of phenyllactic acid from phenylalanine via lactic acid bacteria (LAB)- derived enzymes and has also demonstrated antifungal and antibacterial properties that may extend bread shelf life

[59],[60], [61]. Similarly, azelaic acid was more abundant in sourdough than yeast breads, which has been reported to exhibit antimicrobial and antioxidant properties that could enhance bread shelf-life and provide bioactive effects [62] . Higher abundance of caffeic acid (CA) was observed in the yeast group compared to sourdough whereas the opposite trend was observed for dihydrocaffeic acid (DHCA), a pattern that may reflect bacterial hydrolysis of CA to DHCA during fermentation [63]. Lastly, kaempferol, an anti-inflammatory and antioxidant compound with potential to improve gut barrier function, was also observed in higher abundance in the sourdough group compared to yeast among the differentiating metabolites [64]. Notably, more variation was observed among sourdough samples than yeast. This could be due in part to the larger number of sourdough samples than yeast samples but may also reflect suggesting variation due to starter culture composition.

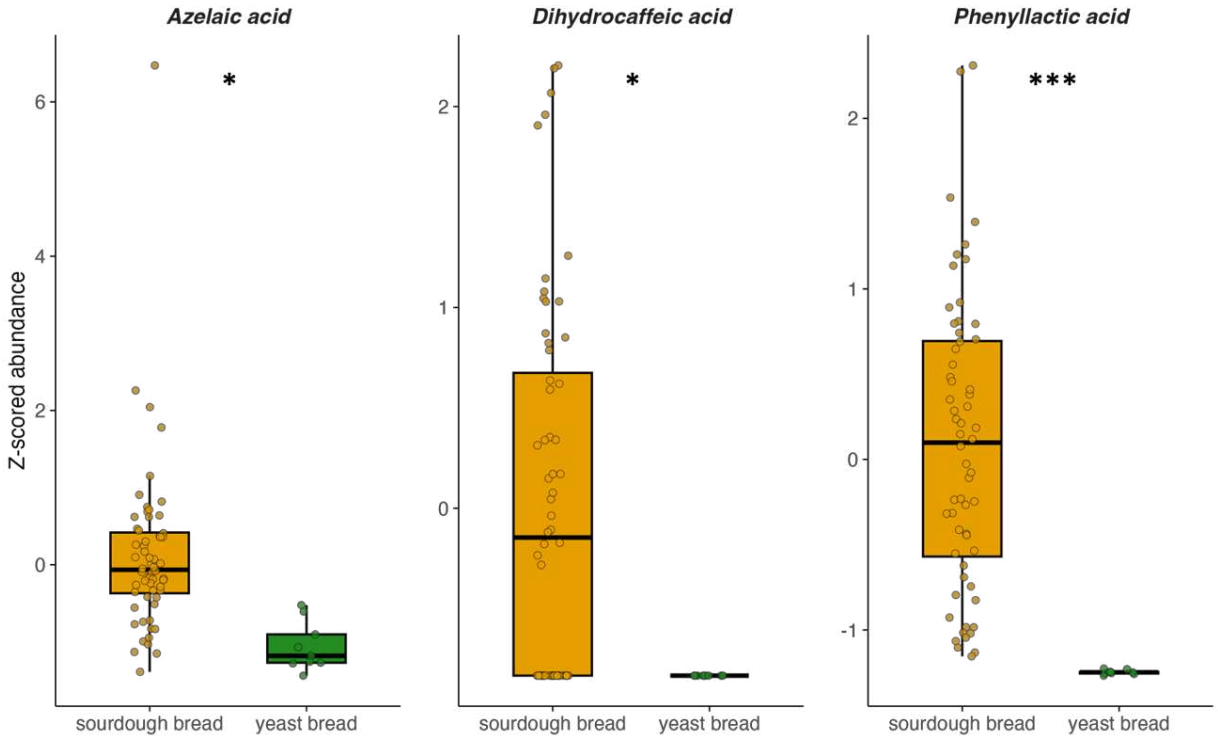

Figure 5. Box plots of 3 of the 356 detect analytes that were significantly different between yeast and sourdough bread. Welch's t test was used to determine significance between sourdough and yeast groups (FDR adjusted p-value, * < 0.05, *** <.001).

Microbial membership of sourdough starter cultures may not solely drive the chemical composition of sourdough bread.

To investigate the factors contributing to the distinct chemical profiles of sourdough samples, we first examined the microbial community structures of the starter cultures. This analysis aims to determine whether the 16S rRNA bacterial taxonomy plays a significant role in shaping the chemical composition of the final bread product. Hierarchical Cluster Analysis (HCA) of the 16S rRNA data revealed three distinct microbial groups (Figure 5a). Bread samples were assigned to these groups to build a PLS-DA model using the metabolomics dataset (Figure 5b).

The fit of the PLS-DA model (R^2X(cumulative) = 0.233, R^2Y(cumulative) = 0.966, Q^2(cumulative) = 0.545) suggests there is good overall fit to the PLSDA however the Q2 value suggests the model is over fit thus the groupings of the 16S analysis may not fully describe the explanation for the metabolomics data of the baked breads.

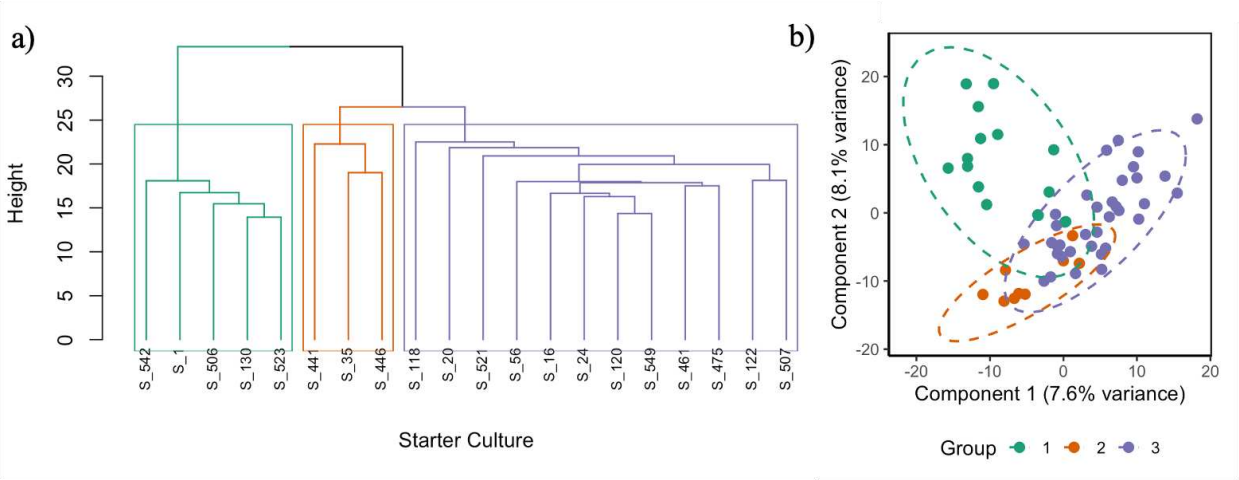

Figure 5. a) Hierarchical Clustering Analysis of 16S rRNA gene sequencing data of sourdough starter cultures (each number represents an individual starter culture). Colors indicate groupings (red = group 1, green = group 2, and blue = group 3). b) PLS-DA scores plot of model trained on sourdough bread samples assigned to the corresponding HCA groups. Colored ellipses represent 95% confidence interval (R^2X(cumulative) = 0.233, R^2Y(cumulative) = 0.966, Q^2(cumulative) = 0.545).

Microbial function may differentially impact chemical composition of sourdough than membership.

While microbial membership provides some insight on the observed chemical variation it is not telling the whole story, it is possible that microbial interactions and metabolic activities within the dough during fermentation could play a more nuanced role in shaping the chemical composition of sourdough breads. To explore this relationship, predicted metabolic functions of the sourdough starter microbiomes were determined using PICRUSt (Figure 5a). Using the predicted functions, HCA revealed 3 groups, notably different than the groups identified based on composition. The sourdough bread samples were assigned to these functional groups to train a new PLS-DA model. There is slight improvement compared to the model based on composition

(Figure 6b; R^2X(cumulative) = 0.234, R^2Y(cumulative) = 0.968, Q^2(cumulative) = 0.667) suggesting the metabolic potential of the microbes could be another predictor of the chemical composition of the bread.

The different hierarchical clustering results likely reflect the functional redundancy between the bacteria in the 16S data, meaning that more than one organism of different taxa can perform the same biochemical function. It has been hypothesized that functional redundancy is a method of stabilization, and the degree of the redundancy is likely determined by the environment of the microbial system[65]. Previous research exploring multiple sourdough starter genomes revealed that the highest number of genes belonged to the largely functional redundant biochemical pathways related to carbohydrate, pyruvate, energy, and nitrogen metabolism [66]. Notably, starter cultures classified in group 1 in the HCA based on functional potential were most distinct from those in the other two groups, suggesting that the microbial composition and functional activity of these cultures may drive a more unique chemical profile of the bread.

It is important to recognize the limitations of PICRUSt, first the outcomes of the analysis are predictions from a reference genome which could exclude rare functions of microbes. Additionally, the prediction is only at the taxa level and cannot contribute strain-specific functionality [67]. Herein this study we use PICRUST as a tool to support the work of future metagenomics sequencing of sourdough samples for direct functional analysis.

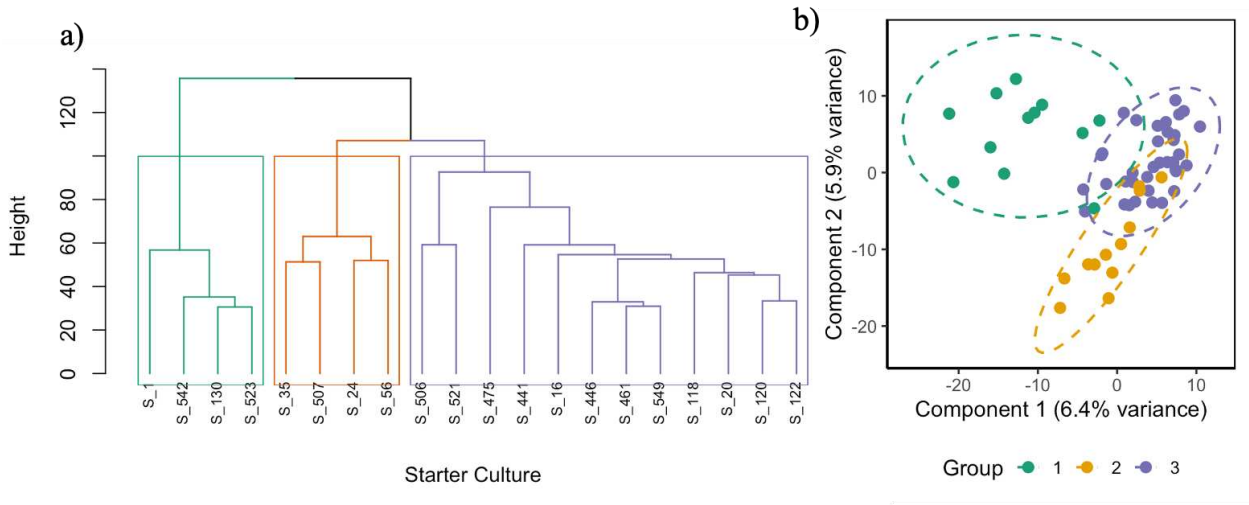

Figure 6. a) Hierarchical Clustering Analysis based on predicted function of sourdough starter microbiomes (each number represents an individual starter culture). Colors indicate groupings and circled compounds represent starter cultures used in further in-vitro analysis b) PLS-DA scores plot of model trained on sourdough bread samples assigned to the corresponding HCA group. Colored ellipses represent 95% confidence interval (R^2X(cumulative) = 0.234, R^2Y(cumulative) = 0.968, Q^2(cumulative) = 0.667).

Radical scavenging of diverse sourdough breads.

The radical scavenging capacity of three sourdough breads produced with different starter cultures was measured to assess their antioxidant potential (Figure 7). Breads made with starter culture 521 exhibited significantly higher Trolox equivalent values compared to the yeast control and starter cultures 24 and 542, indicating greater antioxidant activity. Notably, all groups were significantly different from one another, highlighting that antioxidant capacity in sourdough bread is strongly influenced by starter culture composition and reflects a highly complex system.

Interestingly, when comparing microbial membership groupings with predicted functional groupings, the functional classifications differed from those based solely on microbial

composition. This discrepancy may be explained by functional redundancy among microbes in groups 2 and 3. When samples were instead separated by predicted functional capacity, potentially related to the production of anti-inflammatory or antioxidant metabolites, group differentiation improved (Table 2).

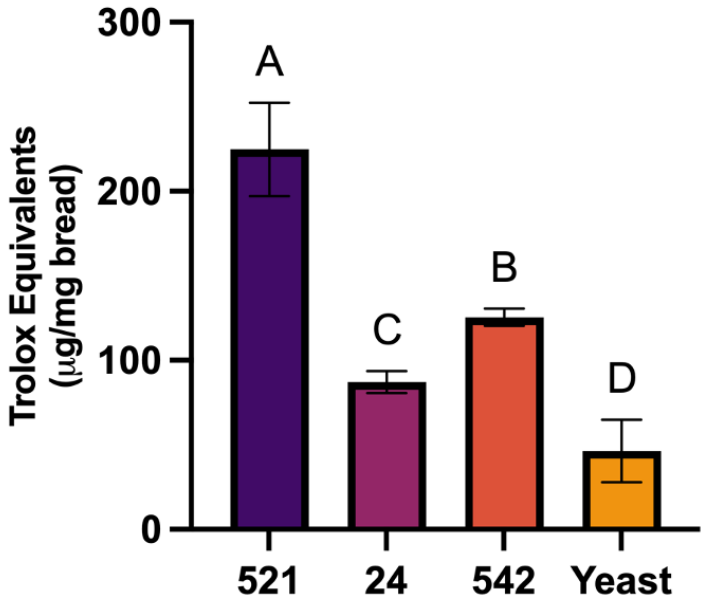

Figure 7. Radical scavenging activity of sourdough breads expressed as Trolox equivalents (µg/mg of bread). A one-way ANOVA revealed significant differences in antioxidant capacity among all leavening agents. Groups that do not share a letter (A–D) are significantly different ($p < 0.05$).

Table 2. Starter cultures used in the radical scavenging activity analysis and their corresponding group assignments based on hierarchical cluster analysis (HCA) of 16S rRNA microbial membership data and predicted functional profiling.

Starter Culture	Microbial Membership Group	Predicted Functional Group
521	1	1
24	3	2

542	3	3

Conclusions

Our analysis of sourdough and yeasts breads and doughs demonstrates distinct chemical profiles between the groups. Our analysis found differential abundances in PUFA compounds that contribute to the separation between breads and dough, noting that food processing, in this case the baking process, changes the chemical composition of the input product making it important to study the food product through such processing. This work also shows sourdough breads to contain compounds that have antioxidant activity and properties that may improve shelf stability, some of these compounds may be bacterially derived supporting the effect of sourdough fermentation to potentially improve the nutritional and bread quality attributes. These findings also explore how microbial composition of the starter cultures may affect the chemical composition of the bread through which we could not define a clear relationship in which we further explored microbial function through predictive tools which may improve our understanding of this relationship. Furthermore, antioxidant capacity of the breads had a differential response based on the sourdough starter culture highlighting the complexities associated with studying microbiomes and the implications they can have on health.

Ultimately, we hope to this work to provide a steppingstone for future studies to elucidate the mechanistic relationship between starter culture microbiomes and bread quality and function, future studies will benefit from deeper metagenomic sequencing. Moreover, longitudinal evaluation of sourdough starter cultures across generations may provide deeper insights into microbial succession and help identify bacterial populations that remain stable over time. In the

future we also hope to further explore the unknown compounds for novel compound discovery of potential microbial derived compounds. Overall, this work sets a road map for the need of further work to better understand the relationship between the microbial make up and function. This work can be used to identify specific microbial organisms that improve nutritional or bread quality for the intentional modification for specific functional and nutritional outcomes of the bread.

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CHAPTER 3: CANNABIDOIL AND CANNABIGEROL ALTER HOST METABOLISM AND GUT MICROBIOTA WITH SEX-SPECIFIC EFFECTS IN MICE

Abstract

Cannabidiol (CBD) and cannabigerol (CBG) are non-psychoactive phytocannabinoids with therapeutic potential, yet their effects on host physiology and interactions with the gut microbiome remain poorly defined. Here, we used a translational mouse model colonized with human-associated microbiotas to investigate how oral CBD and CBG intervention influences microbial composition, host metabolism, and intestinal protein expression. Both phytocannabinoids modulated microbial diversity and specific taxa in a sex-dependent manner. Higher bioavailability was observed for CBD relative to CBG, with females showing higher plasma CBD and CBG concentrations compared to males. Males treated with CBD had elevated anandamide (AEA) levels, potentially reflecting inhibition of fatty acid amide hydrolase, consistent with upregulation of serine protease inhibitors observed in ileal proteomes. Females also accumulated more CBD in fat than males, suggesting adipose storage and slow release into circulation. Collectively, these findings demonstrate sex-specific interactions between phytocannabinoids, the endocannabinoid system, and the gut microbiome, underscoring the importance of considering biological sex in cannabinoid research and informing personalized therapeutic strategies.

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Background

The cannabis plant synthesizes over 120 distinct phytocannabinoids (PCs), including the non-psychoactive compounds cannabidiol (CBD) and cannabigerol (CBG), as well as the well-known psychoactive compound Δ9-tetrahydrocannabinol (THC) [68]. Although THC has historically dominated cannabis research, interest has increasingly shifted toward CBD and CBG due to their therapeutic potential [69], [70]. These compounds have been investigated for the treatment of neurological conditions such as epilepsy, anxiety, and depression, as well as for their anti-inflammatory properties [71]. Nevertheless, this field remains in its infancy, and further evaluation is needed to define their efficacy, safety, and mechanisms of action in a diverse model.

Phytocannabinoids exert many of their effects through the endocannabinoid system (ECS), a highly conserved lipid-signaling network that regulates physiological processes including appetite, metabolism, inflammation, and stress (Balezina et al., 2021; Bielawiec et al., 2020). The ECS comprises endogenous cannabinoids (ECs), their metabolic enzymes, and two main receptors, CB1 and CB2 [68]. The primary ECs, 2-arachidonoylglycerol (2-AG) and anandamide (AEA), are synthesized on demand in response to cellular stimuli such as Ca^{2+} influx or G-protein couple receptor activation and act as retrograde messengers to maintain homeostasis (Bouchet & Ingram, 2020;Alger 2004; Balezina et al., 2021). AEA is a partial agonist at CB1, whereas 2-AG binds with lower affinity but exhibits full agonist efficacy [75]. Their activity is further regulated by metabolic enzymes, membrane transporters, and intracellular carriers, owing to their poor aqueous solubility [76]. Exogenous cannabinoids like CBD and CBG also engage with the ECS and have been reported to act as partial agonists at CB1 and CB2 [77].

Emerging evidence suggests a bidirectional relationship between the endocannabinoid system (ECS) and gut health. For example, 2-AG and other endocannabinoid analogs can strengthen epithelial barrier integrity and reduce intestinal inflammation, while AEA levels have been linked both positively and negatively to inflammatory bowel disease (IBD), likely reflecting differences in disease phenotype, tissue type, or methodology [78], [79], [80], [81]. Although research on CBD and CBG is limited, preclinical and in vitro studies indicate potential benefits: CBD reduces intestinal inflammation and improves barrier function in colitis models (Romano et al., 2013;Alhamoruni et al., 2010), while CBG may modulate cytokine signaling and oxidative stress [84]. These effects may involve bidirectional interactions with the colonic microbiome, which in turn influence the bioactivity of phytocannabinoids. Further mechanistic and clinical studies are needed to clarify the ECS's role in gut health and the factors driving individual variability [85].

Historically, both clinical trials and preclinical models have disproportionately focused on male subjects, leaving a critical gap in our understanding of how females respond to diets, nutrients, and supplements [86], [87]. Furthermore, emerging evidence indicates that the ECS is influenced by biological sex [76]. Research in a murine model showed fluctuations in cannabinoid receptor expression across the estrus cycle [88]. Although mechanisms remain incompletely defined, sex differences in ECS signaling appear to arise from both organizational and activational effects of hormones such as estradiol, which may contribute to sexually dimorphic response to PCs [89]. Riebe et al., (2010) reported reduced CB1 receptor binding in the hypothalamus, but increased binding in the amygdala of female rats relative to males and ovariectomized females. Similarly, estrogen has been linked with decreased CB1 receptor expression and increased expression of the catabolic enzyme FAAH, which degrades AEA [91].

This may help explain reports of lower circulating AEA levels in females across the lifespan [92]. Moreover, sex-specific differences in the physiological and behavioral effects of phytocannabinoids have been observed [93], [94], possibly mediated through the ECS. Collectively, these findings underscore the need for deeper investigation into the sex-dependent mechanism in both phyto- and endocannabinoid biology.

Despite these findings, few studies have investigated how sex and the gut microbiome interact to shape the biological response to phytocannabinoids in a translational model. To address this gap, we utilized a human-microbiota murine model to evaluate the effects of biological sex on: (1) the impact of orally administered CBD and CBG on circulating plasma levels of PCs and ECs; (2) their deposition in subcutaneous fat to assess retention; (3) changes in gut function assessed via exploratory ileum proteomics; and (4) compositional shifts in the gut microbiome. Our findings reveal sex-dependent differences in CBD and CBG metabolism, lipid storage, and modulation of the gut microbiome. These results underscore the importance of including sex as a biological variable in cannabinoid research. As therapeutic use of phytocannabinoids expands, understanding sex-based differences in bioavailability, metabolism, and gut responses will be crucial for developing effective, personalized interventions.

Methods

Experimental Model

Germ-free C57BL/6J mice were bred at the University of Colorado-Anschutz Medical Campus Gnotobiotic Facility (n=36; 18 males, 18 females) (Figure 1). Mice were randomized in a balanced fashion where equal numbers of both sexes received a fecal microbiota transplant

(FMT) from two human donors (microbiome 1, n=18; microbiome 2, n=18). These donor materials were previously analyzed and selected for their distinct microbiota profiles (Trikha et al. 2021). After stabilization of the transplanted microbiomes following FMT, mice were transferred to the Lab Animal Research (LAR) facility at Colorado State University where they underwent a 10-day acclimation period. Mice were individually housed with a 12/12 light cycle and an average room temperature of 73°F. Pre-intervention body weight and food intake were tracked. After completion of the acclimation period the 8–10-week-old mice were equally divided and randomized by sex and microbiota inoculum into one of the three intervention groups: CBD, CBG, or Placebo. Litter sizes resulted in two cohorts of mice, 16 in the first and 20 in the second. Baseline body weight and food intake was recorded, and feces were collected prior to cannabinoid administration. For 29 days, mice received 100 mL of a 5% formulation containing either CBD, CBG, or a placebo. The CBD and CBG were administered as water-soluble nano-emulsions, while the placebo consisted of a carrier emulsion containing 5% medium-chain triglyceride (MCT) oil. All formulations were supplied by Caliper Foods (Commerce City, CO). Double purity testing and verification with a third-party state licensed lab (SC Labs, Denver, CO) was conducted for all three formulations. Daily administration was pipetted onto one-half of a Mini Baked Oyster Cracker (Kroger, USA; Avg daily intake of ½ cracker: 0.2 g, 0.97 kcal, 0.02 g fat, 2.36 mg sodium, 0.15 g carbs, 0.01 g protein, 0.01 mg iron). Crackers were placed in an open and clear area of each cage, free of obstruction and animals were observed for confirmation of consumption. Animals did not display notable aversion to this mode of administration, with the cracker typically being consumed with 30 min of placement in the cage. An oral dose was administered between 8-10am daily through voluntary consumption of ½ of an oyster cracker containing the phytocannabinoid or placebo preparation. Feces were

collected at baseline, week 2, and week 4. Mice were euthanized by exsanguination (cardiac puncture) under anesthesia at the end of week 4 and tissues were weighed (Supplemental Table 1). In addition to plasma, adipose tissue and ileal samples were excised and frozen in liquid nitrogen for downstream proteomic and metabolomic analysis, and all samples were stored at -80°C until analyzed.

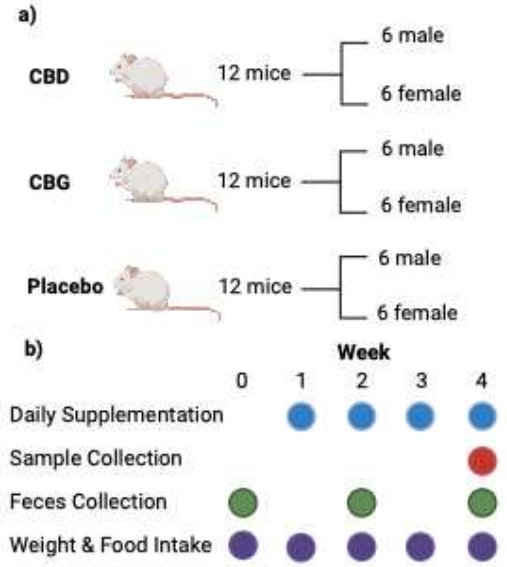

Figure 1. a) Experimental design of the murine model. Female and male mice received a fecal Microbiome Transplant (FMT) from a two distinct donors Microbiome 1 and Microbiome 2. Mice received intervention of either CBD, CBG, or Placebo b) Time points for each type of sample collection, daily supplementation, sample collection, feces collection, and weight and food intake. Image created in BioRender.

Plasma and Subcutaneous Adipose Tissue Targeted Metabolomics

Materials for analysis

LC-MS grade water, methanol, ethanol, formic acid, and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA). ACS grade toluene was obtained from Sigma-Aldrich

(St. Louis, MO). Phytocannabinoid standards, AEA, 2-arachidonoyl glycerol (2-AG), and 1-arachidonoyl glycerol (1-AG), along with the internal standard 2-AG-d5 were purchased from Cayman Chemical (Ann Arbor, MI). CBD, CBG, and the internal standards cannabidiol-d3 (CBD-d3) were obtained by Cerilliant (Round Rock, TX) and cannabigerol-d3 (CBG-d3) was obtained from Sigma (St. Louis, MO).

Sample Preparation

Samples were thawed overnight at 4 °C. Aliquots of 100 µL plasma were added to 500 µL ACS-grade toluene spiked with internal standards AEA-d_4, CBD-d_3, 2-AG-d_5 at concentrations of 5.00, 30.0, and 3.00 ng/ml. For fat analysis, 100 +/- 30 mg of fat was weighed in a glass vial and the internal standard CBG-d^3 was also spiked at a concentration 10 ng/mL in toluene. Samples were vortexed for 20 min at 4 °C, followed by the addition of 500 µL LC-MS grade water. Samples incubated at –80 °C for 2 hours then centrifuged at 2500 x g for 20 min. For the fat, samples were incubated at –80 °C for 3 hrs then centrifuged at 2500 x g for 45 min. From the top organic layer, 300 µL were transferred to a new vial and dried under nitrogen using a positive pressure manifold. Dried samples were reconstituted in 50 µL of a 1:1 (v/v) mixture of acetonitrile and methanol. The reconstituted fat samples were stored at -80 °C overnight. Before analysis, samples were transferred to inserts and centrifuged again for 5 min. Pooled quality controls (QCs) were prepared by taking 10 µL from each plasma extraction after reconstitution and pooling them. Extraction blanks were prepared using 100 µL of LCMS grade water instead of plasma and 100 µL of toluene for fat.

Standard curves were prepared using spiked analytes and internal standards at concentrations ranging from 0.122 to 250 ng/mL. These standards underwent the same extraction process as the samples. For plasma, 100 µL of solution was extracted following the procedure described above. For fat samples, a similar extraction was performed, but 400 µL of spiked toluene was added to maintain equivalent concentrations. All samples were vortexed and centrifuged prior to analysis.

Instrumental analysis

UPLC-MS/MS analysis was performed using a PerkinElmer Q-Sight 420 triple quadrupole mass spectrometer for multiplexed targeted analysis of phyto- and endocannabinoids. Samples were injected onto an LX50 UHPLC system (PerkinElmer, Shelton, CT) equipped with a BEH C18 Acquity UPLC column (2.1 × 100 mm, 1.7 µm; Waters Corporation, Milford, MA). The column was maintained at 60 °C. The mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The gradient elution program started at 41% mobile phase A and 59% B at 0.00 min, then to 30% A and 70% B at 6.00 minute, followed by 1% A and 99% B at 6.10 min, and to 41% A and 59% B from 7.20 to 12.00 min. The flow rate was 500 µL/min, and the injection volume was 5 µL. Detection was performed using electrospray ionization in positive mode and multiple reaction monitoring (MRM). Mass spectrometry settings included: drying gas temperature 120 °C, hot surface induced desolvation (HSID) temperature 200 °C, nebulizer gas flow 250, MRM transitions for each compound were optimized by direct infusion of authentic standard. Multiple transitions were monitored for the compounds (Table 1).

Table 1. Retention time, precursor, quantifier ion (m/z), Collision Energy (CE) for the quantifier, Entrance Voltage (EV) for the quantifier, qualifier ion (m/z), CE for the qualifier, and EV for the qualifier for each phyto- and endo- cannabinoid analyzed in the method.

Compound Name	*Abbr*	*Retention Time, min*	*Precursor (m/z) [M+H]*	*Quantifier (m/z)*	*CE, V*	*EV, V*	*Qualifier (m/z)*	*CE, V*	*EV, V*
Cannabinoid	CBD	3.215	315.1	193.2	-29	35	123.1, 181.2	-43, -24	31, 31
Cannabigerol	CBG	3.168	317.1	193.2	-24	15	123.2	-47	19
Arachidonoyl ethanolamide	AEA	3.467	348.2	79.2	-86	19	287.4, 91.1, 62.2	-3, -57, -26	1, 22, 15
2-Arachidonoyl glycerol	2-AG	4.335	379.3	91.1	-66	36	287.3, 67.2, 55.1, 269.3	-3, -57, -69, -12	38, 35, 36, 38
1-Arachidonoyl glycerol	1-AG	4.582	379.3	91.1	-66	35	287.3, 67.2, 55.1, 269.3	-3, -57, -69, -12	38, 35, 36, 39
Cannabinoid-d3	CBD-d3		318.2	196.4	-31	23			
Cannabigerol-d3	CBG-d3								
2-Arachidonoyl glycerol-d5	2-AG-d5		384.3	91.1	-104	36			
Arachidonoyl ethanolamide-d4	AEA-d4		352.2	66.1	-24	24			

Data Processing

Peak areas were normalized to the appropriate internal standards and quantified based on linear regression of the external calibration curve. The limits of detection (LOD) and quantitation (LOQ) were calculated as three times and ten times the standard deviation of the blank divided by the slope of the calibration curve, respectively. Calculated concentrations in plasma samples were then further normalized by the dilution factor of the sample processing. Concentrations in the fat samples were further normalized by the aliquot mass and for total mass of subcutaneous fat. Values below the LOD were imputed as half the LOD for statistical analyses [95].

Proteomics analysis of ileum tissue

Sample preparation

Samples were thawed from –80 °C and transferred to 2 mL reinforced tissue grinding tubes (Fisher Scientific). Each was combined with 1 mL of tissue grinding buffer (Thermo Lysis Buffer containing 2% SDS and 1× HALT Protease Inhibitor Cocktail). Homogenization was followed by centrifugation at 8,000×g for 10 min. Supernatants were collected in 400 µL aliquots and precipitated with 1.6 mL cold acetone overnight at –20 °C. Protein pellets were washed three times with 300 µL cold acetone, vortexed, and centrifuged each time. Pellets were air-dried and resolubilized using the EasyPrep Mini MS Sample Kit lysis buffer (Thermo Fisher Scientific). Total protein concentrations were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Absorbance was measured at 550 nm, and concentrations were calculated using a bovine serum albumin standard curve (quadratic fit) in Microplate Manager 45 software (Bio-Rad). Proteins were digested using the EasyPep Mini MS Sample Prep Kit according to manufacturer protocol.

Data Acquisition

Peptides were separated via reverse-phase chromatography with water + 0.1% formic acid (A) and 80% acetonitrile + 0.1% formic acid (B). A 1 µg sample was purified and enriched on an online enrichment column (Thermo Scientific PepMap Neo C18, 5 mm × 300 µm). Separation was performed on a Vanquish Neo UHPLC system (Thermo Scientific) using an IonOpticks Aurora Ultimate Gen 3 C18 column (75 µm × 25 cm, 1.7 µm particle size) at 45 °C with a flow rate of 300 nL/min. The gradient elution program started at 41% mobile phase A and 59% B at 0.00 minutes, shifted to 30% A and 70% B at 6.00 minutes, rapidly transitioned to 1% A and 99% B at 6.10 minutes, and returned to 41% A and 59% B from 7.20 to 12.00 minutes.

Eluted peptides were analyzed using an Orbitrap Eclipse mass spectrometer (Thermo Scientific) with a Nanospray Flex ion source. MS spectra were collected from 375–2000 m/z in positive mode. Ions with charges of +2 or higher were selected for MS/MS with dynamic exclusion (1 MS/MS event per precursor, 60 s exclusion). MS1 detection was performed in Fourier Transform mode; MS/MS detection was conducted in ion trap mode using higher-energy collisional dissociation at 30% normalized collision energy with centroid data.

Data Processing and Normalization

Data were processed using Proteome Discoverer (Thermo Scientific). A precursor detector node (S/N = 1.5) was used to identify additional precursors in chimeric spectra. Spectra were searched using Sequest HT with dynamic methionine oxidation and fixed cysteine carbamidomethylation.

Fragment ion mass tolerance was 0.6 Da, parent ion tolerance was 10 ppm. Results were rescored using INFERYS (deep learning-based fragment ion prediction). Peptide spectral matches were validated via Percolator, with an FDR threshold of ≤1%. Protein identification required at least one unique peptide. Normalization was performed using peptide-spectrum match signal relative to the total MS/MS signal, followed by within-injection relative abundance normalization.

16S rRNA for Analysis of Gut Microbiome

Mouse fecal DNA was extracted using the MPBio FastDNA SPIN Kit (Pittsburgh, PA) following the manufacturer's protocol with an additional wash before binding. The gut microbiome was sequenced using 16S rRNA sequencing of the V4 region (primers 515F–806R) with forward primers containing 12 bp Golay barcodes. Libraries were sequenced on an Illumina MiSeq (250 -cycle kit, 15% PhiX spike) at the Colorado State University Next Generation Sequencing Core. Amplicon data were processed in QIIME2 (v2024.2; Bolyen et al., 2019) using the DADA2 pipeline [97]. Taxonomic classification was performed using the SILVA database (v138; Quast et al., 2013).

Statistical Analysis

Plasma and Fat Metabolomics

Metabolite concentrations were imported into R version 4.4.3, R.Studio (Version 2024.12.1+563) for statistical analysis and data visualization. Normality was assessed for each metabolite using QQ plots and residuals. Metabolites with normally distributed data were analyzed using ANOVA followed by Tukey's post hoc test; sex-specific differences were subsequently evaluated with t-tests and false discovery rate (FDR) correction.

Metabolites that were not normally distributed were log-transformed, and normality was reassessed. If normality was achieved after transformation, the log-transformed data underwent ANOVA with Tukey's post hoc test, followed by sex-specific t-tests with FDR correction. Metabolites that remained non-normal after log transformation were analyzed using an Aligned Rank Transformation (ART) ANOVA with Tukey's post hoc comparisons. Sex-specific differences for these metabolites were evaluated using Wilcoxon rank-sum tests with FDR correction.

For the fat metabolomics dataset, outliers were removed using interquartile range (IQR)–based filtering within each intervention–sex group for each analyte. Specifically, for each group, the first quartile (Q1) and third quartile (Q3) were calculated, the IQR was defined as Q3 – Q1, and values falling outside Q1 – 1.5 × IQR to Q3 + 1.5 × IQR were excluded.

Proteomics

Following data normalization, protein accession numbers and associated intensity values were loaded into R for statistical analysis. Proteins that were detected in less than 50% of the samples were discarded. Then missing values were replaced with 20% of the minimum intensity of that

protein for statistical analysis. A three-way Analysis of Variance (ANOVA) was performed on the log2 intensity by sex, intervention, microbiome. False Discovery Rate (FDR) was performed and filtered with a p value less than or equal to 0.2. Proteins were then evaluated by significance by sex and intervention.

16S rRNA Sequencing

Microbiome analyses were conducted in R (v4.4.1) using MicrobiomeAnalystR (v2.0; *Chong et al., 2020)* and Maaslin2 (v1.18.0;Mallick et al., 2021). Features with >4 counts in at least 10% of samples were retained. Read counts were normalized by total sum scaling. Alpha diversity (Shannon Index) was tested using the Kruskal–Wallis test with FDR correction. Beta diversity was calculated using Bray–Curtis dissimilarity, visualized by Principal Coordinate Analysis (PCoA), and evaluated with PERMANOVA (Adonis). Differentially abundant taxa were identified using Maaslin2 with a compound Poisson linear model (CPLM) and zero-inflated negative binomial (ZINB), applying a significance threshold of $q < 0.1$. Graphs and tables were generated in R.

Results

Metabolomics

Intervention Interactions in Plasma

Five cannabinoids, CBD, CBG, AEA, 1-AG, and 2-AG, were quantified in plasma using a validated UPLC-MS/MS method with deuterated internal standards and surrogate internal standards. All calibration curves exhibited linearity across the quantification range ($R^2 > 0.993$) for quantifier ions and intraassay CVs were below 10% for pooled QCs.

Plasma concentrations of CBD were significantly higher in the CBD intervention group compared to both the placebo and CBG groups, which were below the limit of detection (LOD) (ANOVA, CBD-CBG p= 5.71 e-07 and CBD-Placebo p = 5.71 e-07, Supplemental Table 2), indicating that only mice receiving the CBD intervention had detectable levels of circulating CBD (Figure 2a). Similarly, CBG concentrations were significantly higher in plasma from mice that received the CBG intervention (ANOVA, CBG-CBD p= 1.5 e-02 and Placebo-CBG p= 1.5 e-02, Supplemental Table 2) compared to the placebo and CBD groups which were both below the LOD (Figure 2b). These findings confirm that the interventions were effective in producing measurable circulating levels of their respective phytocannabinoids. Among the endocannabinoids measured, only AEA levels differed significantly between the CBD and CBG intervention groups, and between the CBD and placebo intervention groups (ANOVA, CBD-CBG p = 0.0111 and CBD-Placebo p= 0.0111, Supplemental Table 2), (Figure 2c).

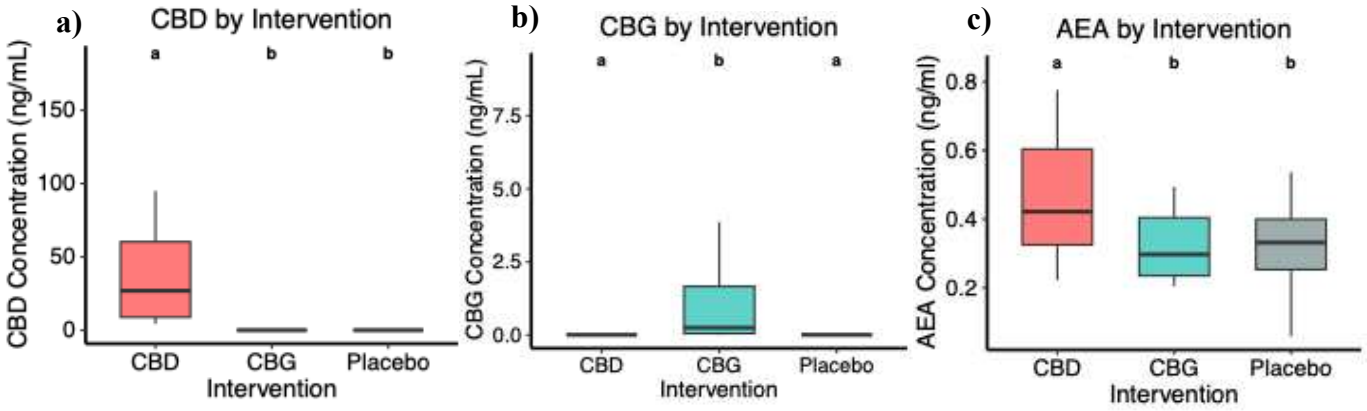

Figure 2. Boxplots showing plasma concentrations of (a) CBD, (b) CBG, and (c) AEA, separated by intervention group (CBD, CBG, or a placebo MCT oil)**.** Significant differences among groups were determined by ANOVA followed by Tukey's post hoc test ($p < 0.05$), with letter annotations denoting group differences. Corresponding p-values are listed in Table S1. Concentrations are expressed in ng/mL of plasma.

Sex-Intervention Interactions in Plasma

Among mice receiving the CBD intervention, the mean plasma concentration of CBD was significantly higher in females compared to males (p=0.0411, Figure 3a, Supplemental Table 2). A similar result was observed with mice receiving the CBG intervention although the difference was not statistically significant (p= 0.126, Figure 3b, Supplemental Table 2). Within the CBD intervention group, AEA was observed to be significantly higher in males compared to females (p= 0.000600, Figure 3c, Supplemental Table 2) and was also higher, but not significant, in males receiving the CBG intervention. No significant sex by intervention interactions were observed for the endocannabinoids 1-AG, and 2-AG.

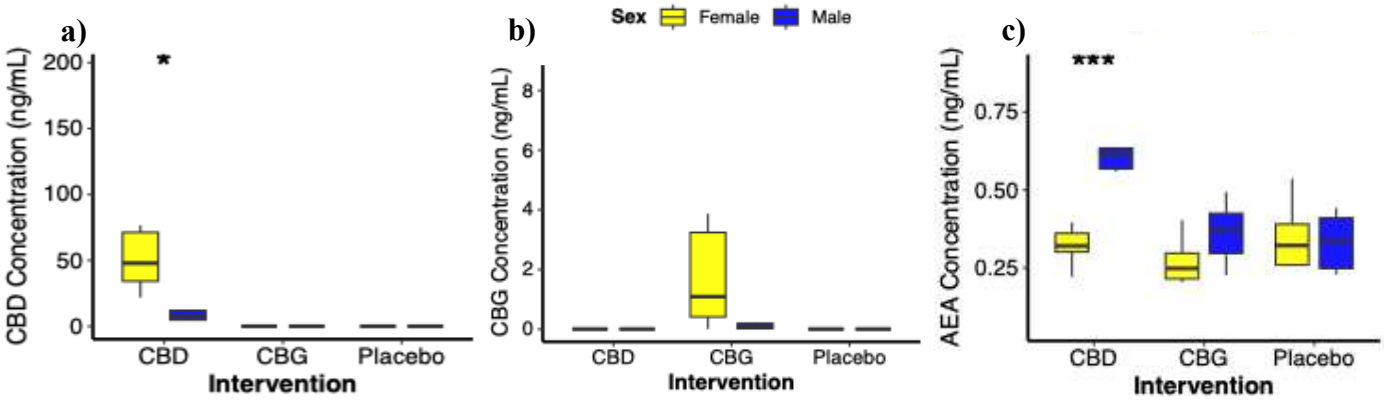

Figure 3. Boxplots of a) CBD, b) CBG, and c) AEA plasma concentration. Results from female animals are represented in yellow (left) and male animals in blue (right). The FDR adjusted p-values between sex are shown in Table S2. Concentrations are expressed in ng/mL of plasma.

Sub-cutaneous Adipose Tissue (SAT) Metabolomics: Sex-Intervention Interactions

Females treated with CBD and CBG exhibited increased SAT tissue, prompting further investigation into the effects of CBD and CBG on SAT (Supplemental Table 1). Among mice receiving the CBD intervention, the amount of CBD in the sub-cutaneous adipose deposition was significantly higher in females compared to males (p= 0.0130, Figure 4a; Supplemental Table 2). A similar result was observed in mice receiving the CBG intervention although the difference was not statistically significant (p= 0.109, Figure 3b, Supplemental Table 2). No significant sex by intervention interaction was observed for the endocannabinoids AEA, 1-AG and 2-AG.

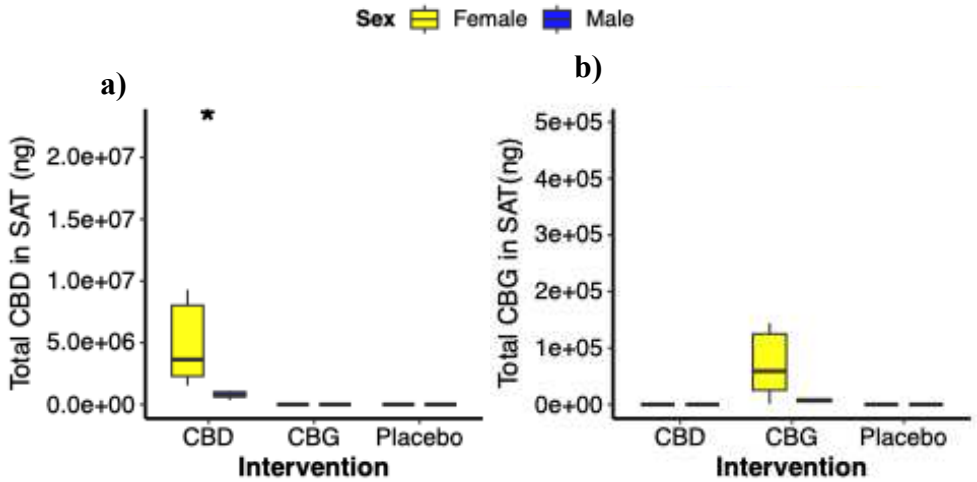

Figure 4. Boxplots of total ng a) CBD and b) CBG in subcutaneous fat (SAT) for each intervention stratified by sex. Female levels are represented in yellow (left) and male levels in blue (right). Wilcoxon rank sum test p-value between groups shown on plot with FDR adjusted p-value.

Proteomics Analysis of Ileum Tissue

In total, 8595 proteins were detected after data processing and filtering. A three-way ANOVA was conducted to identify proteins with significantly different abundances across intervention, sex, microbiome, and their interactions, revealing 26 differentially expressed proteins (Supplemental data, Figure S1). Molecular functions of the proteins identified include binding, enzyme activity, inhibitor activity, receptor activity, signaling/regulation, and structural/other (Figure 5a).

Scaled abundance of the 20 proteins found to be significantly different by three-way ANOVA (sex, intervention, and/or sex × intervention interaction) are presented (Figure 5b), focusing on the CBD intervention versus placebo, stratified by sex. This focus was motivated by the significant intervention effects identified in plasma and fat analyses (Figures 3 & 4). Among the proteins differing by sex, major urinary proteins associated with male pheromones were more abundant in males. Additionally, proteins involved in the inhibition of serine proteases were significantly higher in males (Figure 5b). Notably, other proteins with serine protease inhibitory functions were trending (although not significant) as more abundant in males compared to females when the entire dataset was examined (Supplemental Figure S2) (Supplemental Information Figure S2a, c, and d). Accession numbers associated with epithelial tight junctions and gut barrier integrity including zonula occludens-1, claudin-1, -3, -4, -5, -12, and occludin were queried in the dataset and were either not detected or were not significant between intervention or sex (Supplemental data).

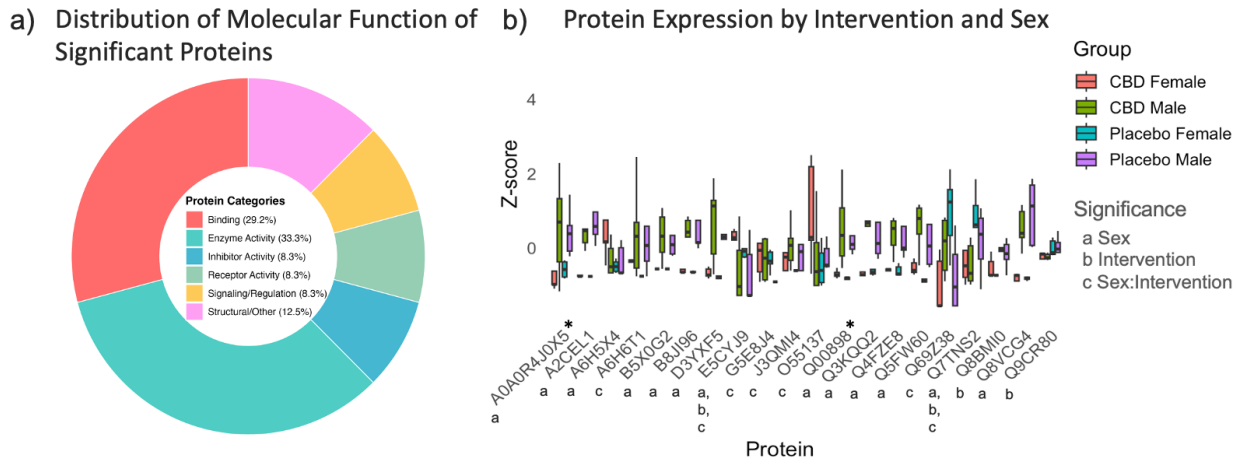

Figure 5. Differential Protein Signatures in Ileum Tissue Identified by 3Way ANOVA. **a)** Donut plot showing the molecular function categories of the 26 proteins identified as significant by the 3-way ANOVA. Functional categories were assigned based on UniProt annotations and manually grouped. The distribution includes binding (29.2%), enzyme activity (33.3%), inhibitor activity (8.3%), receptor activity (8.3%), signaling/regulation (8.3%), and structural/other functions (12.5%). **b)** Boxplots of protein expression for proteins significant for **(a)** sex, **(b)** intervention, or **(c)** the sex-by-intervention interaction, shown across CBD females, CBD males, placebo females, and placebo males. The asterisks (*) denote those proteins with serine protease inhibitor activity.

16S rRNA Sequencing Results

16S sequencing data were analyzed to evaluate intervention effects (Poehlein et.al. 2025). Alpha and beta diversity did not differ significantly by sex within intervention group after four weeks. However, specific taxa exhibited significant differences between intervention and sex groups (Figure 6). Both the zero-inflated negative binomial (ZINB) and compound Poisson linear model (CPLM) functions in MaAsLin2 identified *Akkermansia.1* as significantly enriched in females receiving the CBD intervention when compared to placebo (ZINB: coef = 6.5, FDR = 0.0004; CPLM: coef = 4.5, FDR = 0.007), with the ZINB model estimating a stronger effect, consistent with zero inflation in this taxon's count distribution (Figure 6A). Among females in the CBG

intervention group compared to placebo, both models identified *Lachnospiraceae NK4A136 group.1* (ZINB: coef = 4.8, FDR = 8×10^{-6}; CPLM: coef = 5.0, FDR = 0.003) and *Lachnospiraceae NK4A136 group.19* (ZINB: coef = 5.2, FDR = 0.002; CPLM: coef = 5.6, FDR = 0.02) as significantly enriched (Figure 6B). In males, CBD intervention compared to placebo was positively associated with *Oscillospiraceae.3* (ZINB: coef = 7.9, FDR = 6.8×10^{-7}; CPLM: coef = 8.0, FDR = 0.003) and *Lachnospiraceae FCS020* (ZINB: coef = 6.0, FDR = 2.3×10^{-17}; CPLM: coef = 6.4, FDR = 0.08) (Figure 6C).

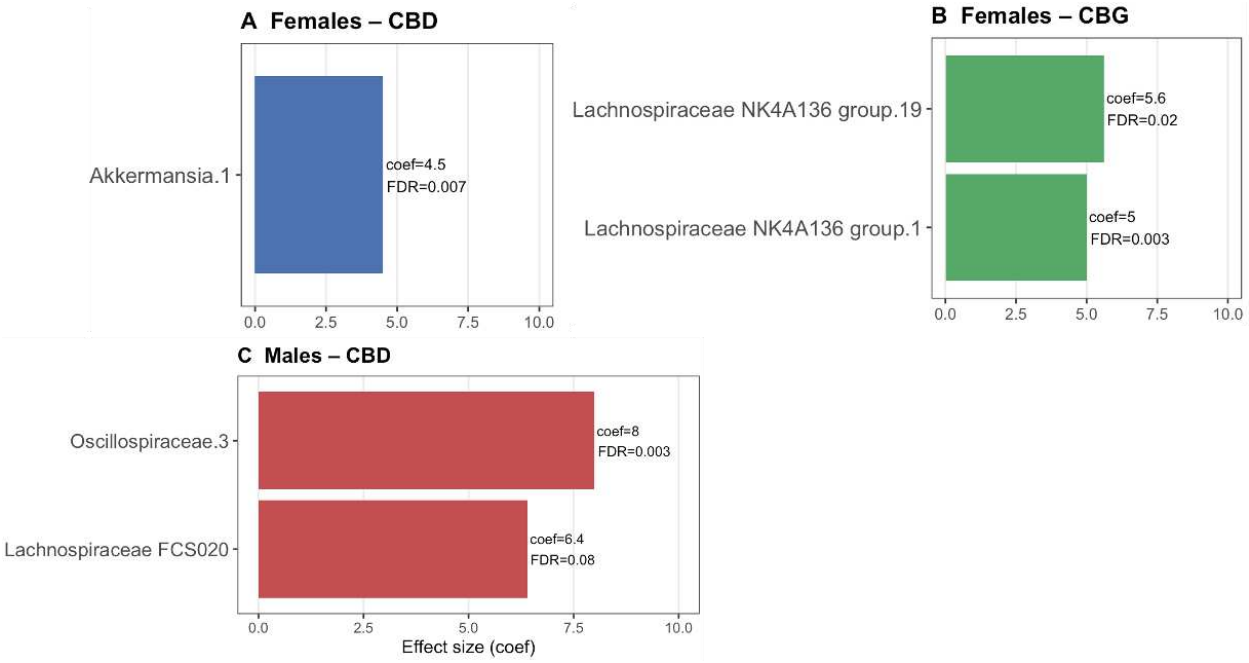

Figure 6. Taxa associations of the CBD and CBG intervention in females and males in reference to placebos of CPLM coefficient values and FDR p-values. A) Females with CBD intervention shows significant levels of Akkermansia.1 (CPLM: coef = 4.5, FDR = 0.007) compared to placebo. B) Females with CBG intervention showed significant levels of identified *Lachnospiraceae NK4A136 group.1* (CPLM: coef = 5.0, FDR = 0.003) and *Lachnospiraceae NK4A136 group.19* (CPLM: coef = 5.6, FDR = 0.02). C) Males with CBD intervention *Oscillospiraceae.3* (CPLM: coef = 8.0, FDR = 0.003) and *Lachnospiraceae FCS020* (CPLM: coef = 6.4, FDR = 0.08).

Discussion

The therapeutic potential of CBD and CBG continues to grow significant interest, despite limited understanding of their effects, particularly on the gut microbiome and how these effects may differ between sexes. This study investigated the sex-specific impacts of four-week CBD, CBG, or placebo MCT oil intervention in human microbiota-associated mice, analyzing circulating and adipose tissue levels of PCs and ECs, the ileal proteome, and the gut microbiota. Our translational model provides critical pre-clinical data to bridge the gap toward clinical applications.

Our results confirm effective intervention delivery, with measurable circulating levels of both phytocannabinoids. Notably, despite an identical dosage, plasma concentrations of CBD were approximately ten-fold higher than those of CBG. This aligns with existing pharmacokinetic data in mice, which reports that orally administered CBG is absorbed more rapidly (T_{max}~ 30 min) but exhibits lower overall bioavailability ($AUC_{0–6h}$ 378 µg*min/mL) compared to CBD (T_{max}~ 60 min, $AUC_{0–6h}$ 57 µg*min/mL) [101]. Our results are consistent with these findings, indicating that CBD achieves higher systemic exposure in this model.

Given the emerging evidence on sex-specific variation in both the response to PC intervention and the ECS [102], we next evaluated plasma levels of PC and ECs by sex to investigate potential differences. We observed significant sex-specific effects in response to CBD intervention, with a similar, though non-significant, trend for CBG. We hypothesized that these differences could be attributed to the sequestration of lipid-soluble phytocannabinoids in subcutaneous adipose tissue (SAT) over the 4-week study period. This hypothesis is supported by previous work from Williams et al., (2021), who found that the time to peak concentration

(T_{max}) for CBD in humans was shorter in individuals with greater fat-free mass, suggesting that body composition is a key determinant of CBD bioavailability and distribution. Our data revealed that female SAT contained significantly higher amount of CBD than male SAT, with a similar trend for CBG. This is physiologically plausible, as females generally have a higher percentage of SAT [104]. The more rapid metabolism of CBG may also explain its less pronounced sex differences, as our sampling may have missed its peak concentration window.

Furthermore, within the CBD intervention group we found that plasma AEA concentrations were significantly higher in males compared to females. This finding may be linked to the inhibitory effect of CBD on FAAH, the primary enzyme responsible for AEA degradation. In a human clinical trial, patients with schizophrenia treated with CBD showed significantly elevated AEA levels, suggesting FAAH inhibition as a likely mechanism [105]. This is consistent with evidence showing that CBD is a more effective FAAH inhibitor than other phytocannabinoids like CBN (Nicoara et al., 2025). The authors propose that CBD's mechanism may involve a complex interaction between the oxidative and hydrolytic pathways of AEA metabolism, though the precise mechanism remains to be fully elucidated.

While our ileal proteome data did not show significant changes in FAAH expression, its activity could be modulated in other tissues. Additionally, the increased abundance of serine protease inhibitors we observed in male ileum suggests a potential for broader regulation of serine hydrolase activity, a class that includes FAAH [106], [107]. Although speculative, this represents a plausible mechanism for the elevated AEA in males warranting further investigation.

Interestingly, we did not observe a significant difference in abundance of detected tight junction proteins, despite previous reports that CBD enhances intestinal barrier integrity [108]. Other tight junction proteins such as the claudins -1, -4, -5, and -12 were not detected in our proteomics analysis, likely due to a sensitivity limitation for low-abundance proteins rather than a true biological absence. These observations are not unexpected as the standard purified diet used in this study should not induce intestinal inflammation. Furthermore, ex vivo analysis of fecal water extracts from these animals showed no changes in trans-epithelial electrical resistance when tested on differentiated Caco-2 monolayers (Poehlein et. al., 2025), consistent with our in vivo proteomics data. Future studies using a high fat or inflammation-inducing model are needed to further evaluate the impact of PCs on functional intestinal host response.

Finally, we assessed the impact of intervention and sex on the gut microbiome. No significant system-level differences in alpha or beta diversity were observed between groups (Poehlein et.al. 2025), suggesting that neither intervention nor sex strongly altered overall microbiome diversity. However, differential abundance analyses (Poehlein et.al. 2025) revealed subtle but notable shifts in specific taxa, reflecting potential intervention- and sex-dependent effects. In the CBD intervention group, we observed a significant enrichment of *Akkermansia*, a genus associated with gut barrier strengthening and improved metabolic health, in females [109], [110]. In males, the *Lachnospiraceae FCS020* group, known for butyrate production, was increased *[111] and strains Oscillospiraceae 3* is a short-chain fatty acid (SCFA) producer and has been associated with positive affects in relation to obesity [112], [113]was also increased compared to placebos. In contrast, CBG intervention led to an enrichment of two *Lachnospiracea NKA136 groups* taxa in females, which are key fermenters of dietary fiber and producers of SCFAs [114]. These findings suggest that while CBD and CBG intervention

did not drastically alter global microbiome diversity, it did induce distinct, sex-specific shifts in key bacterial taxa with functional implications for gut health, particularly through the modulation of mucin-degrading and SCFA-producing populations.

Conclusion

In summary, the results of this study provide novel insight into the sex-specific bidirectional interactions between non-psychoactive PCs, the ECS, and the gut microbiome. Our data reveal that biological sex is a critical determinant of circulating PC and EC, which could impact implications for their therapeutic application.

We identified pronounced sex-dependent differences in the plasma concentrations of CBD and CBG following intervention. The higher concentrations of PCs sequestered in the subcutaneous adipose tissue (SAT) of females suggests a model of gradual release that may underlie the observed differential plasma PC concentration between sexes. Furthermore, the elevated circulating levels of AEA in males within the CBD intervention group points to a sex-dependent modulation of the ECS. While FAAH expression was not significantly different in the ileum, its potential regulation by the observed increase serine protease inhibitors in males presents a compelling, tissue-specific modulation that merits further investigation.

Although the four-week intervention with CBD or CBG did not alter global gut microbiome diversity, our analysis revealed significant sex-by-intervention interactions at the taxonomic level. The enrichment of distinct, health-associated bacteria, such as *Akkermansia* in females in the CBD intervention group and butyrate-producers in males in the CBD intervention group and females in the CBG intervention group, indicates that PCs induce subtle but functionally

important, sex-specific shifts in microbial ecology. Future studies employing whole genome metagenomic sequencing will be crucial to elucidate the functional pathways

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