Dietary Protein's Effect on the Gut Microbiome and Its Metabolites: Protocol Description and Virtual Recruitment Efforts for a Remote Randomized Controlled Trial

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Abstract:

Fiber's effect on our gut microbes has been studied extensively, while knowledge of the effect of protein is sparse. Colonic putrefaction of dietary protein is known to produce several potentially detrimental compounds. This randomized controlled trial (RCT) uses a pre- and post-study design to investigate the effect of consuming higher levels of dietary protein on the gut microbiota and the metabolites it produces. Here, we describe our virtual recruitment efforts and RCT protocol. Potential participants lived throughout the contiguous US. Several recruitment methods were employed, of which email reached the largest group of people (approximately 9,100) and generated a 0.3% conversion of contacts to participants, which exceeded the number of participants needed per group based on our power analysis. Forty-five people were enrolled and randomized to the pea group and forty-two to the whey group. The participants are now completing the study. They will consume their usual dietary intake and add 50 gms of a whey or pea protein supplement for seven days. A fecal sample will be collected before and seven days after increasing their protein intake. Gut microbes and fecal metabolites will be identified. The results will demonstrate the effect of a high level of protein consumption on the gut microbiota and the metabolites it produces.

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1. Introduction

Most intestinal microbes reside in the colon, where they ferment and process food that has not been digested and absorbed in the small intestine. Dietary fiber is one food substance that reaches the colon untouched by small intestine digestive processes. The interrelationship between colonic microbes and fiber has been extensively studied, and recent studies have demonstrated this relationship's profound effect on our health (Clemente et al., 2012; Cronin et al., 2021; Glowacki & Martens, 2020; Wilson et al., 2020). However, our diet also contains other dietary constituents, like lipids and protein, which reach the colonic microbes but have not been extensively studied (Bartlett & Kleiner, 2022).

Often, Western culture promotes a high-protein diet, particularly animal protein. For example, a high-protein diet has been touted as a successful weight-loss strategy (Moon & Koh, 2020); athletes often increase their protein intake to develop muscle (Phillips & Van Loon, 2011); and a higher protein intake is recommended for older people to slow age-related sarcopenia (Baum et al., 2016). More than 50% of Americans consume more than twice their Recommended Dietary Allowance (RDA) for protein (Bartlett & Kleiner, 2022; U.S. Department of Health and Human Services and U.S. Department of Agriculture, 2015). The amount of protein consumed, the digestibility of that protein, and the meal matrix can affect the amount of protein entering the large intestine (Yao et al., 2016). Studies have estimated that 12-18 g of dietary protein reaches the colon per day based on ileostomy studies with protein intake closer to the RDA (Evenepoel et al., 1999; Rodriguez-Romero et al., 2022; Yao et al., 2016).

Dietary fiber is also an essential nutrient. The American diet does not provide the Dietary Reference Intake (DRI) (Institute of Medicine, 2006) for fiber and averages 15.9 g of fiber per day (King et al., 2012). With the abundance of protein in the Western diet, it is possible that more protein than fiber could reach the colon.

Our previous study investigated the gut microbiota and metabolome of self-identified muscle-builders who reported either using or not using protein supplements (Byerley et al., 2022). Participants'

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protein intake averaged 117.6 ± 11.8 (mean ± SEM, no protein supplement) vs. 169.3 ± 17.6 (protein supplement), more than two times the RDA, while their dietary fiber intake was 18.9 ± 2.1 (no protein supplement) vs. 27.3 ± 3.1 (protein supplement) (Byerley et al., 2022). It is possible both groups had more protein than fiber present in the colon for the microbes to ferment.

Our novel finding was a difference in nitrogen and purine metabolism between the two groups' fecal matter. The fecal metabolomic data found significant differences in several metabolic pathways interconnected with nitrogen metabolism. Interestingly, the group that reported using protein supplements had a higher protein intake and increased allantoin in their feces, a microbial purine breakdown product (Byerley et al., 2022). Food rich in protein can also be high in purines, which may be why allantoin increased in the group reporting supplement use. Reducing one's protein intake to reduce purine intake is the dietary treatment for gout (Choi et al., 2004), which is caused by uric acid crystals, the breakdown product of purines and the immediate precursor of allantoin (Roman, 2023).

Protein and purines are nitrogen sources in the diet, although protein contributes more nitrogen than purines. Their gastrointestinal metabolic fate is shown in Figure 1. First, protein is denatured in the stomach and then enters the small intestine, where it is broken down into peptides and amino acids. About 89 to 95% of the amino acids are absorbed in the small intestine (Bos et al., 2005; Silvester & Cummings, 1995). Next, the unabsorbed peptides and amino acids enter our colon, where millions of bacteria break them down. Bacterial fermentation produces branch-chain fatty acids (BCFAs), ammonia, hydrogen sulfide, phenolic (p-cresol) and indole compounds, amines, and/or polyamines (Diether & Willing, 2019). Some products are absorbed, while others only interact with the colonic epithelium. Hydrogen sulfide, p-cresol, and ammonia are detrimental to the colonic epithelium (Blachier et al., 2022), while indole compounds maintain the barrier function of the epithelial cells (Davila et al., 2013).

Some dietary purines are absorbed in the small intestine while the remaining purines reach the colon, where bacteria catabolize them into xanthine, uric acid, and allantoin (Yamauchi et al., 2020)

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(Figure 1). Allantoin is believed to provide carbon and nitrogen sources for bacterial production of other chemical compounds. Whether colonic allantoin is absorbed has not been clearly established. Additionally, it is unknown if the host excretes allantoin, produced by free radical conversion of uric acid, into the gastrointestinal tract. The cycling, metabolic fate, or consequences of allantoin have not been well-characterized (Cicero et al., 2023).

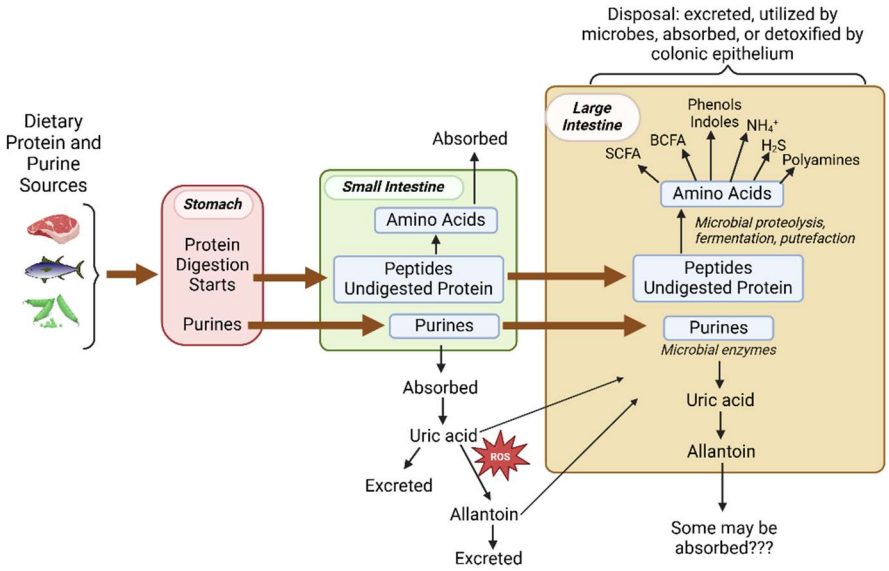

Figure 1. The metabolic fate of dietary protein and purine sources in the gastrointestinal tract. BCFA = branch-chain fatty acids; SCFA = short-chain fatty acids; H_2S = hydrogen sulfide; NH_4 = ammonia; ROS = reactive oxygen species.

Both the amount of protein consumed and the source of protein (plant vs. animal) influence colonic protein fermentation; however, it is still unclear which one, plant or animal, or both, has the greater effect. Also, the interaction within the gut between protein and fiber has not been well-established (Jackson et al., 2024). Eleven human studies have examined how adding protein to the diet affects the gut microbiome (Table 1) (Beaumont et al., 2017; Cotillard et al., 2013; David et al., 2014; Duncan et al., 2007; Fluitman et al., 2023; Mitchell et al., 2020; Moreno-Perez et al., 2018; Russell et al., 2011; Salonen

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et al., 2014; Walker et al., 2011; Windey et al., 2012). Six of these studies had a parallel design (Beaumont et al., 2017; Fluitman et al., 2023; Mitchell et al., 2020; Moreno-Perez et al., 2018; Salonen et al., 2014; Walker et al., 2011), four were crossover studies (David et al., 2014; Duncan et al., 2007; Russell et al., 2011; Windey et al., 2012), and one was non-randomized (Cotillard et al., 2013). The number of subjects per study varied from 12 to 49 (Beaumont et al., 2017; Cotillard et al., 2013; David et al., 2014; Duncan et al., 2007; Fluitman et al., 2023; Mitchell et al., 2020; Moreno-Perez et al., 2018; Russell et al., 2011; Salonen et al., 2014; Walker et al., 2011; Windey et al., 2012), and protein intake ranged from 50 to 144 g/day (Beaumont et al., 2017; Cotillard et al., 2013; David et al., 2014; Duncan et al., 2007; Fluitman et al., 2023; Mitchell et al., 2020; Moreno-Perez et al., 2018; Russell et al., 2011; Salonen et al., 2014; Walker et al., 2011; Windey et al., 2012). The supplementation period ranged from 4 days (David et al., 2014) to 6 months (Fluitman et al., 2023). David et al. (2014) found that gut microbiota changed within 24 hours and documented that these changes persisted for the four days that participants continued the diet. Afterward, they altered their diet, and within 24 hours, the gut microbiota reflected that change. These observations informed our decision to increase daily protein intake for a shorter duration, specifically seven days. We also balanced the study duration with participant compliance, as they would be adding 50 grams of protein daily to their usual intake.

For the eleven studies detailed in Table 1, the different proteins used included animal only (Moreno-Perez et al., 2018), a combination of plant and animal protein (Beaumont et al., 2017; Cotillard et al., 2013; David et al., 2014; Fluitman et al., 2023; Mitchell et al., 2020; Salonen et al., 2014; Windey et al., 2012), or plant protein alone (David et al., 2014). Four studies that gave a mixture of plant and animal protein for 2-3 weeks (Beaumont et al., 2017), 10 weeks (Mitchell et al., 2020), or 6 months (Fluitman et al., 2023) in parallel study arms or crossover design (Windey et al., 2012) did not find a detectable difference in the gut microbiota composition, while seven studies reported that protein-induced differences in the microbiota (Cotillard et al., 2013; David et al., 2014; Duncan et al., 2007; Moreno-Perez

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et al., 2018; Russell et al., 2011; Walker et al., 2011; Windey et al., 2012). However, gut microbiota composition varied greatly, with the Bacteroidetes, Actinobacteria, or Firmicutes phyla most affected (Cotillard et al., 2013; David et al., 2014; Duncan et al., 2007; Moreno-Perez et al., 2018; Russell et al., 2011; Walker et al., 2011; Windey et al., 2012). This is also supported by a review by Blachier et al. (Blachier et al., 2019). Based on these studies, we included both a plant and animal protein arm in our study.

High interindividual variability in intestinal gut microbiota is well documented, making interpretations of human studies difficult (Salonen et al., 2014; Thursby & Juge, 2017; Walker et al., 2011). To reduce this variability, we use a pre- and post-study design approach to examine the effect of increasing dietary protein and purine intake on bacterial composition, fermentation, and metabolite production.

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154 Table 1. Study design, subject number, protein source, quantity, and supplementation duration for studies investigating the effect of protein on
155 the gut microbiome.

Study Design	Subject Number	Type of Protein	Quantity	Supplement Time	Citation
Parallel	12	MP[1] + APS[2]	Casein: 82 g/d to 143 g/d	21 days	(Beaumont et al., 2017)
	13	MP + PPS[3]	Isolated soy protein: 96 g/ d to 160 g/d soy		
	13	MP	Maltodextrin control: 92 g /d to 75 g/d maltodextrin		
Parallel	14	MP	High protein (144 g/d), medium carbohydrate weight loss diet	21 days	(Salonen et al., 2014)
Parallel	14	MP	High protein (144 g/d), medium carbohydrate weight loss diet	21 days	(Walker et al., 2011)
Parallel	12	MP + APS	Whey isolate and beef hydrolysate: 10 g of each added to usual intake; 149 g/d	70 days	(Moreno-Perez et al., 2018)
	12	MP	Maltodextrin control: 129 g/d		
Parallel	14	MP	RDA[4]	10 weeks	(Mitchell et al., 2020)
	14	MP	2x RDA		
Parallel	43	MP	<1.0 g protein/kg adjusted[5] body weight/day	6 months	(Fluitman et al., 2023)
	47	MP	1.2 g protein/kg adjusted body weight/day		
Crossover	20	MP	Normal Protein (NP): 74 g/d	14 days for each diet, with 14 days between HP and LP diets	(Windey et al., 2012)
		MP + APS	High Protein (HP) (whey protein, 20 g/day): 124 g/d (>25% of energy needs derived from protein)		
		MP	Low Protein (LP): 50 g/d (9% of energy needs derived from protein)		
Crossover	17	MP	Maintenance diet: 85 g/d	7 days maintenance diet followed by 28 days of each diet crossed over	(Russell et al., 2011)
		MP	High protein, moderate carbohydrate: 139 g/d		
		MP	High protein, low carbohydrate: 137 g/d		
Crossover	19	MP	Maintenance (M): 94 g/d	3 days M followed by 28 days HPMC or HPLC	(Duncan et al., 2007)
		MP	High protein, medium carbohydrate (HPMC): 127 g/d		

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		MP	High protein, low carbohydrate (HPLC): 120 g/d		
Crossover	10	MP + APS MP + PPS	Animal protein: 133 g/d (30% of energy) Plant protein: 42 g/d (10% of energy)	4 days	(David et al., 2014)
Non-randomized	49	MP MP	Low calorie, high protein: 97 g/d Weight maintenance: 76 g/d	6 weeks	(Cotillard et al., 2013)

[1]MP = Mixed protein. Subjects consumed a base diet of both animal and plant protein sources. The contribution of each protein source is not provided in the paper.
[2]APS = Animal protein supplement
[3]PPS = Plant protein supplement
[4]RDA = Recommended Dietary Allowance (0.8 g protein per kg body weight)
[5]Adjusted to put the participant in their nearest healthy BMI range

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Protein-rich diets tend to contain larger quantities of purines than diets less rich in protein (Choi et al., 2004). While purines are ubiquitous, some foods, such as meat, seafood, and a few vegetables, are higher in purines than other foods (Kaneko et al., 2014). Inside the human body, purines are broken down to uric acid, a causative agent for gout, but humans lack uricase, the enzyme that converts uric acid to allantoin. In the gut, microbes break down uric acid to allantoin, but its metabolic fate and health implications are unknown. In a non-human model, Yamauchi et al. (Yamauchi et al., 2020) found elevated levels of allantoin in fruit flies (whole body and feces) after feeding a high purine diet (adenosine) for 1 or 4 days. This study also reported that the high purine diet significantly shortened the lifespan of the flies.

Research Question and Objectives: The overall research question asks how dietary protein impacts our gut microbiota and the metabolites it produces using a pre- and post-randomized controlled trial (RCT) study design, which is described in this paper along with the results of our virtual recruiting of healthy young and middle-aged male and female adult participants. There are three study objectives:

- Determine if increasing dietary protein intake increases allantoin production in the colon.
- Establish a model to examine the effect of dietary protein and purines on the gut microbiota and metabolites.
- Identify gut microbiota and metabolite changes associated with animal (whey) or plant (pea) protein intake.

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2. Materials and Methods.

2.1. Study Design

The study is a pre- and post-randomized controlled trial (Figure 2). This design was selected to minimize inter-individual variability in the gut microbiome, often observed among individuals who do not cohabitate. Individuals living in the same household have a more similar microbiota than individuals living in different communities (Song et al., 2013). Using each participant as his or her control minimizes interindividual fecal microbiota differences due to diet, exercise, geographical location, sleep, etc. (Parizadeh & Arrieta, 2023). The study design and timeline are shown in Figure 2.

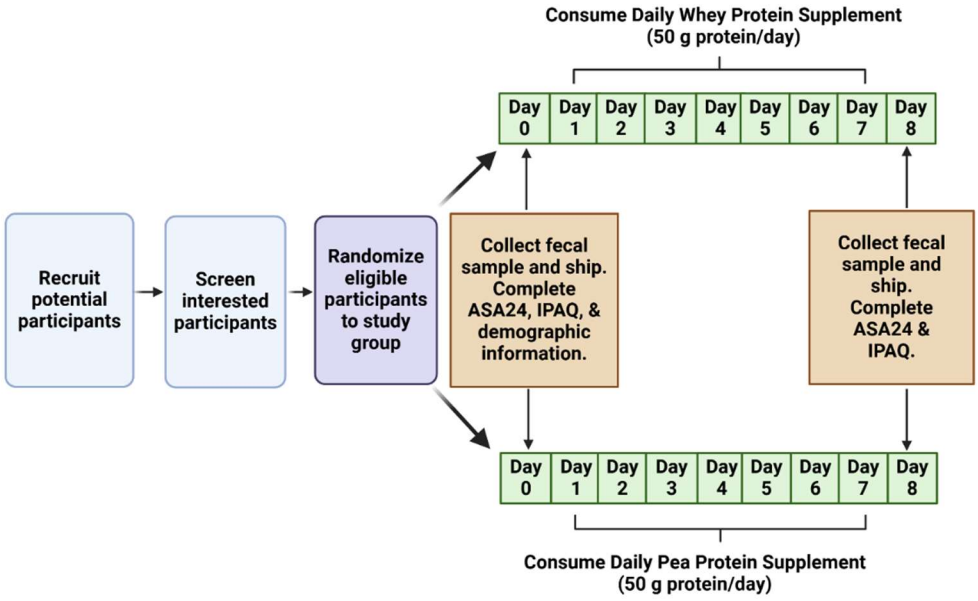

Figure 2. Study design and timeline.

2.2. Protection of Human Subjects

To ensure the protection of human subjects, this research was conducted in accordance with the ethical principles in the Declaration of Helsinki, which is overseen by the Institutional Review Board (IRB). The IRBs at the American Public University System (APUS, 2022-075) and Louisiana State University

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Health Sciences Center New Orleans (LSUHSC-NO, 5008) reviewed and approved all protocols. The study is registered at Clinicaltrials.gov (NCT06677333).

2.3. Sample Size Calculations

A power analysis was conducted based on the effect of protein on allantoin from our previous observational study (Byerley et al., 2022). Allantoin was the metabolite with the highest VIP (Variable Importance in Performance) score (Byerley et al., 2022). To detect a 2-fold difference in metabolites between groups, with an alpha of 0.05 and 80% power, it was determined that at least 15 participants per group were needed.

2.4. Study setting

The study will be conducted remotely; in other words, participants will complete the study in their homes located anywhere in the contiguous USA. This allowed participants to continue their regular daily routine.

2.5. Consent

Human Subject and Health Insurance Portability and Accountability Act (HIPAA) consents were obtained electronically before the potential participant completed the participant screening survey.

2.6. Recruitment

Most of our recruitment efforts focused on individuals associated with APUS, which is entirely online and has a large student, faculty, and staff population. Participants were recruited using four methods: email, personal communication, social media, and posted advertisements. A recruitment email with study details, a URL link, and a QR code for the participant interest survey was sent to students

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within APUS's Sports and Health Sciences, Athletic Development Management, Sport Management, and eSports programs. Participants were also recruited by personal communication (face-to-face, phone, or email) within the researcher's circle of family, neighbors, and friends. If the person indicated they were interested in participating, they were given the survey URL link and QR code to complete the participant interest survey. An advertisement containing a brief description of the study, as well as the URL link and QR code, was developed. This was posted on social media sites, such as LinkedIn and Facebook. APUS has three internal community boards on which the advertisement was posted. One board was specific for the graduate students in the Sports and Health Sciences and Sport Management programs, and the other two community boards are dedicated to APUS staff and faculty. The same advertisement was posted on each. Finally, this same advertisement was printed and posted at a community gym.

2.7. Screening

Inclusion/exclusion selection criteria were developed by reviewing similar peer-reviewed published studies for their selection criteria. Once set, a patient interest survey was developed, which asked questions specific to the exclusion criteria shown in Table 2. Briefly, the study required participants to be 1) healthy male and female adults, 2) not taking any prescribed medicines for chronic diseases such as diabetes, hypertension, anxiety, depression, or GI-related diseases, and 3) not diagnosed with cancer or GI conditions.

Table 2. Criteria for excluding participants.

Exclusion Criteria
Younger than 25 years
Prebiotics in the last week
Probiotics in the last week
Prescription medications other than oral contraceptives

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Antibiotics sometime in the last three months
Diarrhea inhibitors in the last week
Laxatives in the last week
Dietary supplement(s)
Diagnosed with cancer
Diagnosed with an inflammatory disease of the GI tract, such as irritable bowel disease
Experienced long-haul COVID fatigue
Physically inactive (<600 METS per week)

2.8. Randomization process

A random number generator equation in Excel (Microsoft Corporation, Redmond, WA) generated a randomized list of protein group assignments. Based on the order in which the screening survey was completed and if a participant passed the screening criteria, they were assigned to the next protein group.

2.9. Protein Supplement

A dried powder protein supplement was selected instead of a whole food, such as a chicken breast, to increase the participant's protein intake. A powdered supplement ensured a protein source with a consistent content that could be easily shipped and did not require special handling by the study participant.

Two different protein supplements were selected: animal (whey) or plant (pea) protein. Dairy is known to be purine-free (Kaneko et al., 2014), while pea is a rich purine source (Kaneko et al., 2014), which is being verified by mass spectrometry. NOW Foods (Bloomingdale, IL) donated three flavors (unflavored, vanilla, and chocolate) for the whey and pea protein supplements.

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For the study, participants will be asked to consume 50 g of protein daily (2 scoops of supplement) on top of their usual dietary intake. This amount was selected because the group reporting protein supplement usage in our previous observational study had a significantly higher protein intake, an average of 52 g more/day, than the non-supplement using group (Byerley et al., 2022).

The supplement can be incorporated into a beverage or food, such as smoothies or cookies, per the participant's preference. To ensure supplement consumption, we 1) will meet with the participant at the beginning of the study to answer any questions; 2) provide three flavors (unflavored, vanilla, and chocolate); 3) provide recipes; 4) send daily text or email messages reminding the participant to consume the supplement; and 5) be available during the study to answer questions.

2.10. Measurement Instruments

The study timeline, shown in Figure 2, details the timing of the following measurement instruments. Before randomization, the participant completed a multi-question participant interest survey administered using Qualtrics (Seattle, WA). The survey asked for basic information such as birth date, address, contact information, height, and weight, as well as responses to the inclusion/exclusion criteria (see Table 2), for example, "Did they consume a prebiotic in the last week?" Finally, they were asked to complete the International Physical Activity Questionnaire (IPAQ) (Craig et al., 2003), which was converted for electronic completion to assess their physical activity level. This information was used to screen potential participants to determine if they qualified and would be randomized. If the individual qualified and was randomized, they were sent an email to determine 1) if they were still interested in participating and 2) if they were responsive to email or text messages. This generated a pool of participants to complete the study. We have completed this portion of the study.

Participants will be sent the study supplies, including the protein supplement, a fecal collection kit, and a stainless-steel shaker tumbler. Participants will be asked to collect a fecal sample and ship it back

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on an ice pack via overnight carrier before consuming the protein supplement for seven days. After they collect their first fecal sample, they will be asked to recall the foods they consumed twenty-four hours before. That data will be collected using the Automated Self-Administered 24-Hour (ASA24® 2020) Dietary Assessment Tool developed by the National Cancer Institute (https://epi.grants.cancer.gov/asa24/). They also will complete the IPAQ electronically and provide information on their fecal collection, such as time and date and whether it was typical. Qualtrics (Seattle, WA) will be used to administer the questionnaire. This same information will be collected when the final fecal sample is collected after consuming the protein supplement for seven days. Upon completing the surveys and collecting the two fecal samples, the participant will receive compensation of one hundred dollars as a gift card.

2.11. Fecal collection

Fecal samples will be collected and shipped twice: 1) before supplement consumption and 2) after seven days of supplement consumption. The BioCollectorTM kit will be used to collect the sample (Byerley et al., 2022). Briefly, a fecal hammock will be attached to the toilet into which the participant will defecate. The hammock will then be placed in a double bagging system (mylar and biohazard bag), put in an insulated shipping box, a frozen freezer pack placed on top, and delivered to an overnight carrier for next morning delivery to Louisiana State University Health Sciences Center (LSUHSC) in New Orleans, LA for processing. Once received, the fecal sample will be aliquoted, and each aliquot will be frozen in liquid nitrogen and stored in a -80° F freezer. One aliquot each (approximately 1 g) will be given to the Microbial Genomics Resource Group (microbiome) and Core Laboratories (metabolomics) at LSUHSC.

2.12. Microbial Community Analysis

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We will use the same process previously published by our group (Byerley et al., 2022). The procedure is briefly described here. Total fecal DNA will be isolated using the QIAamp PowerFecal DNA Kit (Qiagen, Germantown, MD, USA) with bead-beating following the manufacturer's instructions. The 16S ribosomal DNA hypervariable region V4 (252 bp) will be PCR-amplified, and the purified amplicons will be sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA). The Microbial Genomics Research Group at LSUHSC will use QIIME2 (Quantitative Insights Into Microbial Ecology, http://qiime.org/), a well-developed free online software, for demultiplexing, quality filtering, OTU picking, taxonomic assignment, phylogenetics, diversity analyses, and visualizations of microbiome data. Greengenes v13.8 will be used for taxonomic classification (McDonald et al., 2012). PICRUSt (http://picrust.github.io/picrust/) and LEfSe (http://huttenhower.sph.harvard.edu/lefse/) will be used to identify the inferred functional capacity of the microbial communities.

2.13. Metabolomics sample extraction

A fecal sample (~ 200 mg) will be transferred into a 2 mL tube and mixed with 75% iced cold methanol (3.6 µL/mg feces) using a vortex and a couple of large ceramic beads. Following homogenization, 322 µL of suspension will be transferred into a new Eppendorf tube, spiked with 5 µL of Ribitol solution (0.2 mg/mL in water) as an internal standard, and consequently mixed with 370 µL H2O, 195 µL methanol (100%) and 504 µL chloroform. The sample will then be vortexed for 2 min, incubated for 10 min at room temperature, and centrifuged at 14,000 x g for 10 min.

For lipids, the lower (chloroform) phase will be transferred into a new Eppendorf tube and dried. For analysis, the sample will be resuspended in 28 µL of 100% isopropanol and spiked with 2 µL of internal standard Splash 2 lipid mix (Avanti).

For polar metabolites, the upper (aqueous) phase will be transferred to a new Eppendorf tube and dried. Then 40 µL methoxyamine hydrochloride (20 mg/mL in Pyridin) will be added, incubated for 2 h at

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37° C, and further derivatized by adding 70 µL N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) mix (1mL MSTFA + 20 µL FAME used for retention index determination). After incubation for 30 minutes at 37° C, samples will be directly analyzed by GC-MS.

2.14. Metabolomics Untargeted LC-timsTOF-MS/MS Analysis

All analyses will be carried out using a Bruker nanoElute2 System (Billerica, MA) coupled to a timsTOF fleX 2 mass spectrometer. The mobile phases will be composed of Solvent A (10 mM ammonium formate, 20% IPA, 30% ACN, 0.1% Agilent Deactivator Additive) and Solvent B (10 mM ammonium formate, 20% IPA, 30% ACN). The lipids will be separated using a C-18 reversed phase PepSep column (0.2 x 150 mm; 1.5 nm particles; Bruker) coupled with the CaptiveSpray ionization source of the mass spectrometer. The flow rate and temperature of the column chamber will be set to 1 µL/min and 50°C. Separation of lipids will be achieved at the following gradient: T=0.0 min: 0% B; T=1.0 min: 50% B; T=1.6 min: 57% B; T=5.0 min: 70% B; T=5.1 min: 94% B; T=6.4 min: 96% B; T=6.5 min: 100% B; T=7.0 min: 100% B; T=7.2 min: 0% B; T=10.0 min: 0% B (column re-equilibration). MS data will be collected in positive, Data Dependent Acquisition (DDA) – PASEF mode under the following conditions: a capillary voltage of 1,500 V; the source temperature was set at 150°C; the dry gas flow was maintained at 3 L/min; acquisition range was 100 – 1,800 m/z. Tims setting will be as follows: 1/K0 Start: 0.55 Vs/cm2; 1/K0 End: 1.90 Vs/cm2; Ramp Time: 100 ms; Accumulation Time: 100 ms; Duty Cycle: 100%; Ramp Rate: 9.43 Hz. Data processing and analysis will be done using mzMine 4.2.0 (open-source software: https://mzmine.github.io/download.html) and its in silico lipid fragment database.

2.15. Metabolomics Targeted LC-TQ-MRM-MS/MS Analysis (Agilent)

All analyses will be carried out using Agilent's 6495 TQ LC/MS System (Santa Clara, CA) consisting of 1290 Infinity II HPLC and 6495 triple quadrupole mass spectrometer with Jet Stream Technology source.

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The mobile phases will be composed of Solvent A (10 mM ammonium formate, 20% IPA, 30% ACN, 0.1% Agilent Deactivator Additive) and Solvent B (10 mM ammonium formate, 20% IPA, 30% ACN). The lipids will be separated on Agilent's C-18 reversed-phase column ZORBAX RRHD Eclipse Plus C18 (100 x 2.1 mm; 1.8 nm particles) coupled with the jet stream ionization source of the mass spectrometer. The flow rate and temperature of the column chamber will be set to 400 µL/min and 45°C. Separation of lipids will be achieved at the following gradient: T=0 min: 15% B; T=2.5 min: 50% B; T=2.6 min: 57% B; T=9.0 min: 70% B; T=9.1 min: 93% B; T=11 min: 96% B; T=11.1 min: 100% B; T=12.0 min: 100% B; T=12.2 min: 15% B; T=16.0 min: 15% B (column re-equilibration). MS data will be collected in both positive and negative modes under the following conditions: Capillary voltage of 3,500 V (positive set point) and 3,000 V (negative set point); nozzle voltage of 1,500 V (positive set point) and 1,500 V (negative set point); iFunnel high-pressure RF of 150 V (positive set point) and 200 V (negative set point); iFunnel low-pressure RF of 60 V (positive set point) and 110 V (negative set point); source and sheath gas temperatures will be set at 150°C and 200°C, respectively; dry gas and sheath gas flows will be maintained 17 L/min and 10 L/min, respectively; nebulizer pressure will be set to 20 psi. Approximately 763 lipids will be targeted using dynamic Multiple Reaction Monitoring (dMRM) with transitions and parameters described in Agilent's application note (Huynh, 2021). Data processing and analysis will be carried out using MassHunter Quantitative Analysis software (version 12.1, Agilent, Santa Clara, CA).

2.16. Metabolomics detection by GC-MS

An Agilent 8890 gas chromatograph (GC) coupled with an Agilent 7250A Quadrupole Time-of-Flight (Q-TOF) Mass Spectrometer (MS) will be used. The GC is configured with a splitless inlet and an Agilent 7650A automatic liquid sampler (ALS) for injection. An Agilent 122-5532G/DB-5ms column will be used throughout the experiment, with helium as the carrier gas at a flow rate of 4 mL/min and nitrogen as the collision gas at a flow rate of 1.5 mL/min. The oven program is as follows: initial temperature 60°C, held

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for 1 minute; ramped at 10°C/min to 325°C, then held for 10 minutes. The inlet temperature will be set to 230°C, with a pressure of 8.231 psi and a septum purge flow maintained at 3 mL/min. The total run time is 37.5 minutes.

The Q-TOF MS provides high-resolution, accurate mass detection, supporting quantitative analyses. The acquisition rate is 5.0 spectra/second, with 1943 transients per spectrum. The emission current is fixed at 5 µA, and electron energy is set to 70 eV. The ion source operates at 4.9 µA. This instrument features a mass range of up to 3,000 m/z, providing high sensitivity and excellent dynamic range, making it suitable for trace-level compound detection. Data acquisition will be conducted using Agilent MassHunter software.

GC-MS data will be processed using MS-DIAL (v.4.9.221218) (Huynh, 2021) (Tsugawa et al., 2015; Tsugawa et al., 2020) for peak detection, identification, and alignment with the following parameters: retention time range of 0–100 minutes; MS1 and MS2 mass range of 0–1,000 Da; MS1 tolerance of 0.5 Da; MS2 tolerance of 0.5 Da; minimum peak height of 500 amplitude for peak detection; retention type set to RI; FAMEs will be used as the RI compound; retention index tolerance of 3,000; and an identification score cut-off of 70%. All other parameters will be set to their default values. Compound identification will be performed using the FiehnLib library (Kind et al., 2009), with retention index values automatically normalized to Fiehn retention indices based on the elution order of fatty acid methyl esters (FAMEs).

2.17. Data collection, management, and analysis

ASA24, IPAQ, and the survey data will be downloaded as CSV files. SAS, SPSS, or R will be used for data consolidation and statistical analysis. This is a pre- and post-test RCT, with protein intake as the intervention and protein formulation (animal or plant) as the treatment. In this design, each participant serves as their own control, and changes in outcomes (before and after protein supplementation) will be

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visualized using boxplots and compared between treatment groups using 2-way ANOVA. The data will be transformed and normalized where appropriate and corrected for multiple testing. Statistical tests will be 2-sided and maintain an overall type I error rate of 0.05.

2.18. Handling of Missing Data

Only participants who provide two valid fecal samples (before and after) will be included in the data analysis. There is the possibility of missing survey data from the date of fecal collection (ASA24 and IPAC), which may be included in analyses as covariates or stratification variables. A missing value from one of these surveys will be replaced with a valid response from the same participant at another time point (which assumes no change for that participant). If a participant has missing data on all surveys, continuous measures will be replaced with median values from completed surveys, and categorical variables will be included as 'unknown.'

2.19. Data Storage

Survey results will be downloaded from Qualtrics as CSV files and uploaded into Excel for viewing and password protection. Analytic datasets will be consolidated in SAS/STAT software, version 9.4 (Cary, NC), and maintained at LSUHSC. All raw data files will be transferred using secure file transfer with data encryption.

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3. Recruitment Results

Figure 3 shows the flow of participants from their initial point of interest to those who were randomized into the study. Undergraduate and graduate students (n=9139) enrolled at the university were contacted twice for a total of 18,278 contacts. From this, 61 (0.3%) participants were randomized.

Personal communication reached about 60 potential participants, of whom nine (15%) qualified for the study and were randomized. Social media yielded approximately 498 views/impressions, of which three people (0.6%) were randomized. The combined exposure from two website posting boards at APUS had the potential to reach 2339 people. Six people (0.3%) were randomized from this. Finally, one person was randomized based on the advertisement displayed in a gym. We are unsure where the other seven people learned about the study.

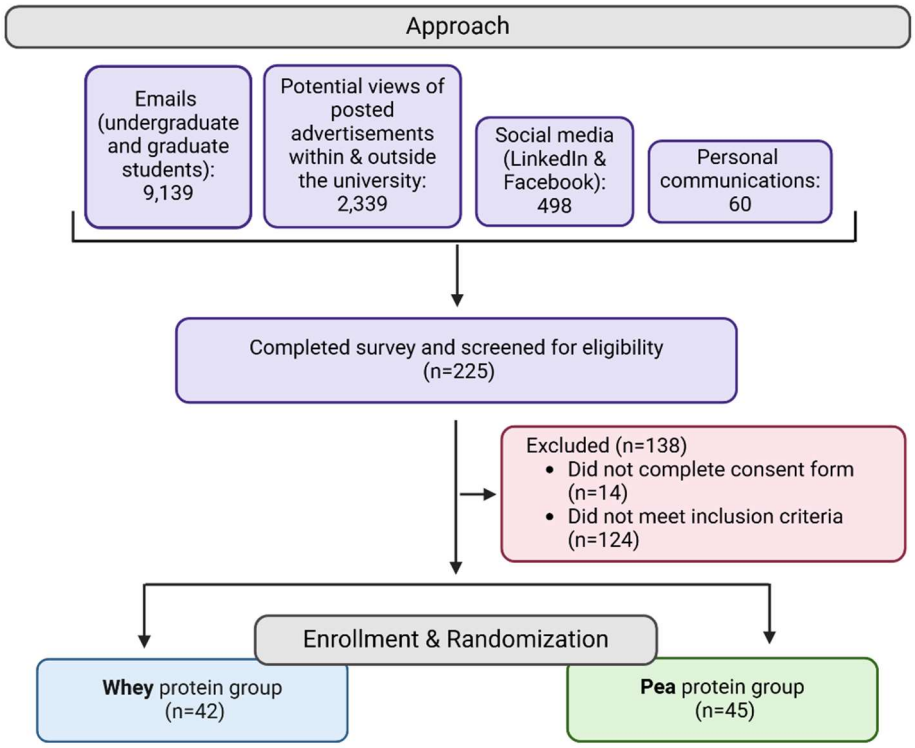

Figure 3. Schematic diagram for recruitment. (Created with BioRender.com).

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Figure 4 shows the geographical location of survey respondents. North Carolina (6%), Texas (5%), Virginia (4%), and California (4%) were the states with the most recruits. No participants were located in Connecticut, Delaware, Idaho, Iowa, Kansas, Maine, Montana, New Mexico, North Dakota, South Dakota, and Vermont.

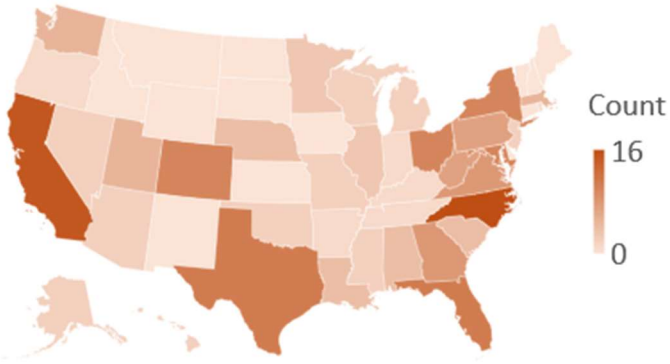

Figure 4. Survey respondents by state.

Table 3 shows the number of disqualified participants based on the exclusion criteria. Prescription drugs were the primary reason many participants were excluded from the study. Next were probiotic and prebiotic consumption in the last week and antibiotics within the last three months. Overnight shipping was unreliable for 8% of the participants who were dropped.

Table 3. Percent of survey respondents and the reason they were disqualified.

	Percentage (n)
Disqualifiers:	
<25 years old	2 (4)
Prebiotics in the last week	7 (15)
Probiotics in the last week	13 (29)
Prescription drugs, excluding OCA	43 (96)
Antibiotics in the last 3 months	5 (12)
Laxatives	2 (4)
Antidiarrheal drugs	1 (3)
Cancer	1 (1)

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GI disorders	3 (7)
Smoked or Vaped	4 (8)
Physical Inactivity	3 (6)
Distractors that needed further inquiry:	
Taking supplements	42 (94)
Taking protein supplement	16 (36)
Food Allergy	4 (10)
Vegetarian lifestyle	3 (6)
Vegan diet	2 (4)
Vegetarian who eats animal products	4 (9)
Diagnosed with long-haul COVID	1 (2)
More than one disqualifier	49 (110)

(n) = number of participants

Participants were asked about their supplement use. If they were consuming a protein supplement, they were asked to continue it during the study. If a vegan or vegetarian were randomized to the whey group and did not want to consume dairy, they were disqualified.

Table 4 provides demographic information on screened participants. More females than males responded to the survey. The average BMI for the survey respondents was slightly overweight, although weight or a BMI greater than 25 did not disqualify someone from the study. According to their IPAQ evaluation, only 3% of interested participants (Table 3) were physically inactive.

Table 4. Demographic information of participants before screening and randomization.

Variable	**Value**
Age (y), mean ± SD, (range)	38 ± 11 (18-70)
Sex, % (n):	
Male	45 (85)
Female	55 (105)
Ethnicity, % (n):	
Hispanic or Latino	11 (22)
Not Hispanic or Latino	89 (172)
Race, % (n):	
American Indian or Alaska Native	2 (3)
Asian	3 (6)
Black or African American	15 (28)
Native Hawaiian or Other Pacific Islander	1 (2)

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White	79 (149)
Height (inches), mean ± SD, (range)	68 ± 1.7 (58-78)
Weight (lbs), mean ± SD, (range)	178 ± 36 (102-290)
BMI, mean ± SD, (range)	27 ± 4 (18-40)
<25, %, (n)	36 (70)
25-30, %, (n)	39 (76)
>30, %, (n)	25 (47)

4. Discussion

This protocol uses a pre- and post-randomized controlled design to determine the effect of dietary protein on colonic microbiota and its metabolites. Other studies investigating the effect of dietary protein on the gut microbiome have employed a crossover design or parallel design, with each group consuming the same protein source for a certain time. We opted to use each participant as their own control to minimize the intervariability in the host microbiome observed across different individuals. Genetically related individuals, whether cohabitating or not, have a more similar gut microbiome than unrelated individuals (Song et al., 2013). Since recruited participants were remote and unrelated, to minimize the variability within the study, a randomized controlled study with pre- and post-sample collection was selected.

With this study, we demonstrate that the number of participants needed to power a RCT can be recruited and successfully ushered through a remote study protocol. However, it took many points of contact to do that. As shown in the CONSORT diagram, more than 12,000 people had to be contacted to have 225 (2%) complete the survey. It is unknown how many people opened and read the emails since that information was unavailable from the university's IT staff. However, most participants were recruited by email. Although this was our most successful way of recruiting, it was still a small percentage of the emails sent. Our study is not the first to use such a strategy. The mSToPS study recruited 2659 participants over ten months entirely by virtual methods (primarily email, but also

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website and direct mail), reaching out to 359,151 Aetna members for an enrollment rate of 0.8% (Baca-Motes et al., 2019). This trial and our study demonstrate that RCT participants can be successfully recruited virtually, but a large audience from which to recruit is needed.

Several studies have successfully recruited participants via social media. Burgess et al. (Burgess et al., 2017) recruited participants via Facebook and Instagram for a 2-group, parallel, single-blinded RCT. Over 30 days, they were able to reach 65,268 people, with 1161 app downloads (2%) and 498 people (<1%) enrolled in their Cool Runnings RCT. In a different study, Ortner et al. (Ortner et al., 2024) used an online campaign that had 886,670 views, reached 309,000 users, and generated 27,814 clicks. From this, 556 users (2%) were screened, and finally, 90 participants enrolled (<1%). For us, social media was not a successful strategy, possibly due to the university and investigator's limited social media presence and bandwidth (a minimal number of followers).

Regardless of which virtual recruitment method is selected, email or social media, a large presence or reachable group of people was necessary because recruitment success was less than two percent. Despite the low conversion rate of contacts to participants, we found that recruiting virtually required less effort, was less intensive, and was more time-efficient than traditional routes.

5. Conclusions

The food we eat contains six nutrient classes, like protein, and many other non-essential chemical compounds, like purines. Proteins and purines are unique in that they contain the element nitrogen. Our research question focuses on how dietary nitrogen sources, like proteins and purines, impact our gut microbiota, the metabolites they produce, and ultimately, our health. Recent studies have demonstrated the profound effect of diet, particularly fiber fermentation, on the gut microbial community. Unfortunately, less attention has been paid to protein and purine fermentation and putrefaction, and many Americans consume more protein than their daily need. This randomized controlled trial focuses

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on healthy young and middle-aged adults, each participant serving as their own control, and fecal microbiota and metabolomic changes measured before and after increasing their dietary protein and purine intake. Understanding this impact is important because protein needs increase as we age, sarcopenia progresses (Walston, 2012), and the relative abundance of the two predominant phyla, Bacteroidetes and Firmicutes, shifts (Walston, 2012). These changes may affect gut protein/purine metabolism and have long-term health outcomes. With this protocol, we have successfully recruited and randomized participants to investigate the effect of a higher dietary protein intake, derived from either a plant or animal source, on the gut microbiome.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board at APUS (protocol #2022-075, 11 November 2022) and the Louisiana State University Health Sciences Center (LSUHSC) (protocol #5008, 2 February 2023).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Research Transparency and Reproducibility: The data presented in this study will be available when the data analysis is completed. If you are interested in obtaining the data, please contact the corresponding author, Lauri Byerley, at lbyerl@lsuhsc.edu.

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Conflicts of Interest: The authors declare there are no conflicts of interest.

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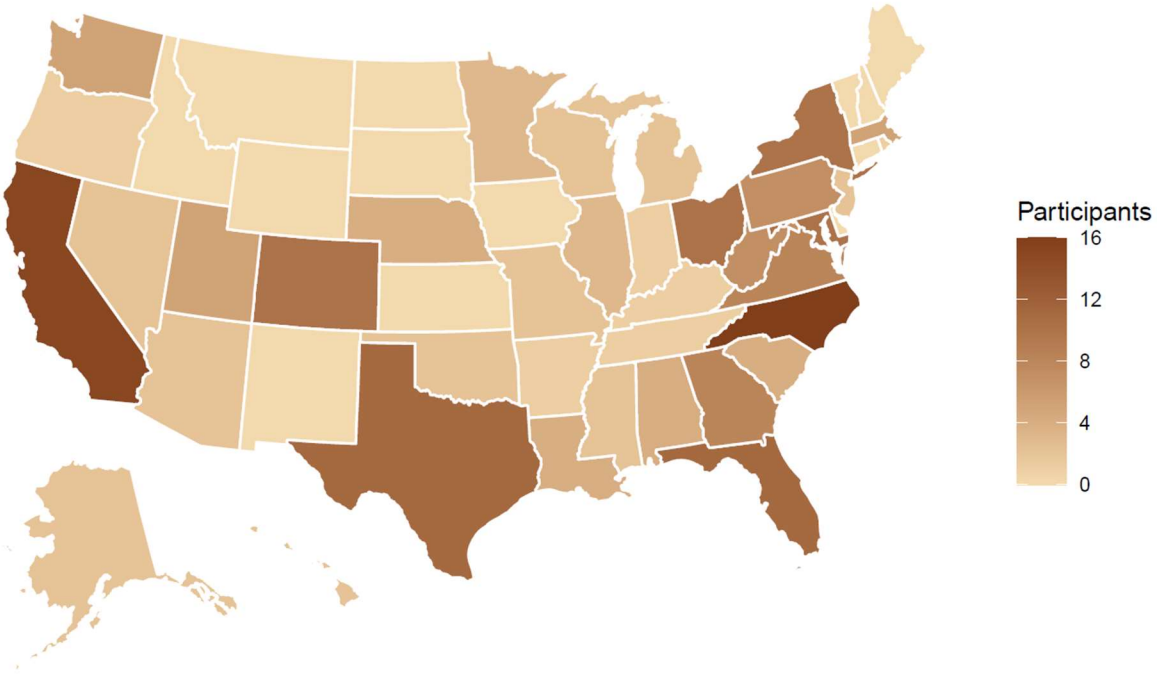
696 **Graphical Abstract**



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