Exploratory Pilot Trial Assessing the Immediate Impact of Animal-based and Plant-based Protein drinks on Blood Parameters

A national, single center, exploratory pilot study

Study Type:	Other Clinical Trial according to ClinO, Chapter 4
Risk Categorisation:	Risk category A
Study Registration:	1. ClinicalTrials.gov (not yet registered) 2. Registration number from the FOPH portal SNCTP (Swiss National Clinical Trial Portal) and, if applicable, other registries and numbers
Sponsor:	Dr. Gommaar D'Hulst, *Oberassistent* Laboratory of Exercise and Health, Department of Health Sciences and Technology, ETH Zürich Schorenstrasse 16, 8603 Schwerzenbach dhulstg@ethz.ch Tel: +41 78 662 44 74
Principal Investigator	Dr. Gommaar D'Hulst, *Oberassistent* Laboratory of Exercise and Health, Department of Health Sciences and Technology, ETH Zürich Schorenstrasse 16, 8603 Schwerzenbach dhulstg@ethz.ch Tel: +41 78 662 44 74
Investigated Intervention:	Role of a plant-based protein drink on amino acid bioavailability in blood
Protocol ID	Study Plant-Based Protein Drink – 2024-00151
Version and Date:	Version 3.0 (dated 22/04/2024)

CONFIDENTIALITY STATEMENT

PROTOCOL SIGNATURE FORM

Study Title	Exploratory Pilot Trial Assessing the Immediate Impact of Animal-based and Plant-based Protein drinks on Blood Parameters
Study ID	Study Plant-Based Protein Drink - 2024-00151

The Sponsor and Principal Investigator have approved the protocol version 3.0 (dated 22/04/2024) and confirm hereby to conduct the study according to the protocol, current version of the World Medical Association Declaration of Helsinki, and ICH-GCP guidelines as well as the local legally applicable requirements.

Sponsor - Principal Investigator:

Name: *Gommaar D'Hulst*

Date: 22-04-2024 _____ Signature:  _

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GLOSSARY OF ABBREVATIONS

AE	*Adverse Event*
ASR	*Annual Safety Repot*
BASEC	*Business Administration System for Ethical Committees*
CRF	*Case Report Form*
CTCAE	*Common Terminology Criteria for Adverse Events*
FADP	*Federal Act on Data Protection (in German: DSG, in French: LPD, in Italian: LPD)*
eCRF	*electronic Case Report Form*
FOPH	*Federal Office of Public Health*
GCP	*Good Clinical Practice*
HRA	*Human Research Act (in German: HFG, in French: LRH, in Italian: LRUm)*
ICH	*International Conference on Harmonisation*
ClinO	*Ordinance on Clinical Trials in Human Research (in German: KlinV, in French: OClin, in Italian: OSRUm)*
SAE	*Serious Adverse Event*
HPLC	*High-Performance Liquid Chromatography*
FSMP	*Food for Special Medical Purposes*
REDCap system	*Web-based Electronic Data Capture system*

1 STUDY SYNOPSIS

Sponsor / Sponsor-Investigator	Dr. Gommaar D'Hulst Laboratory of Exercise and Health Department of Health Sciences and Technology ETH Zürich Schorenstrasse 16, 8603 Schwerzenbach dhulstg@ethz.ch +41 78 662 44 74
Study Title	Exploratory Pilot Trial Assessing the Immediate Impact of Animal-based and Plant-based Protein drinks on Blood Parameters
Short Title / Study ID	Study Plant-Based Protein Drink
Protocol Version and Date	Version 3.0 (dated 22/04/2024)
Study Registration	ClinicalTrials.gov - TBD
Study Category and Rationale	This clinical study qualifies as Category A because it involves only minimal risks to the participants, primarily associated with routine blood sampling, a standard and non-invasive procedure. The interventions include consumption of already marketed and food-grade products, thus not introducing novel or unknown risks. Additionally, the study's objective is to assess nutritional properties rather than to explore therapeutic effects, aligning it with the criteria for low-risk, non-therapeutic research typically categorized under Category A.
Background and Rationale	Addressing environmental concerns, our team has developed a unique beverage from plant-based proteins, aimed at improving bioavailability. This is crucial, as low bioavailability in plant proteins can lead to malnutrition, particularly in the elderly. With an increasing demand for plant-based options among the aging population, our study will compare this new drink's nutritional and amino acid bioavailability with traditional animal-based products. Our goal is to provide a sustainable choice that maintains the nutritional advantages typically found in animal proteins.
Risk / Benefit Assessment	Risks: - Minor discomfort and risks associated with blood sampling - Potential allergic reactions to ingredients in the drinks Benefits: - Advancing knowledge in plant-based nutrition. - Potential development of a nutritionally equivalent plant-based alternative to animal proteins. - Contribution to sustainable food practices.
Objective(s)	The objective of the study is to quantitatively assess the bioavailability of leucine in the blood following the consumption of a novel animal -and plant-based protein drink. This assessment will be compared to the bioavailability observed after consuming a commercially available animal-based protein drink. Leucine is the rate-limiting amino acid that drives protein synthesis in muscle and is associated with preservation of muscle mass during aging and inactivity. the study aims to determine whether the plant-based drink can match or exceed the bioavailability of amino acids typically seen in animal protein sources, thereby offering a viable nutritional alternative for those seeking plant-based diets.
Endpoint(s)	Leucine bioavailability in blood (Area Under the Curve)
Study Design	Exploratory, single center, randomized, single-blinded, cross-over controlled pilot study with three arms
Statistical Considerations	Repeated-measures ANOVA, sample size calculation is on the expected effect size based on previous literature, the desired level of statistical power (80%), and the significance level (0.05).
Inclusion- / Exclusion Criteria	Inclusion criteria: male and female, 18-40 years, signed written informed consent Exclusion criteria: smoking history, cardiovascular diseases , BMI > 35 kg/m2, allergies: Soy, Peas, Milk, Lactose, Fructose

Number of Participants with Rationale	The power analysis was informed by a precedent human study (PMID: 36615694) that established average leucine AUC values to be 20,000 (with a standard deviation of 2,000) for plant-based drinks and 28,000 (with an updated standard deviation of 6,100) for animal-based drinks (whey). The mean difference between plant and animal drinks was identified as 8,000 units from this research. This study was performed in men and women, with no differences in leucine AUC between genders. Adjusting for the crossover design, the power analysis indicated that approximately 9-10 participants would suffice to achieve the desired statistical power of 0.80 and an alpha level of 0.05. This sample size estimation directly reflects the enhanced efficiency and reduced variability afforded by the crossover design, as compared to a traditional parallel-group RCT, in discerning significant differences in leucine AUC among the dietary interventions examined. We will recruit 12 participants to account for dropouts (10-20%). Calculations have been done in python
Study Intervention	Participants will be administered three different drinks in a randomized crossover order. These drinks are: - Animal-based A: A commercially available high protein drink, based on animal proteins (Fresubin Protein Energy). - Animal-based B: A homemade drink containing animal-based proteins, using a specific animal-based protein isolate mix. - Plant-based A: A homemade drink containing plant-based proteins, formulated to have the same macronutrient content as the animal-based drinks. Participants will arrive fasted at the lab. After a 60 min seated rest, a baseline blood sample will be taken 30 minutes prior to consuming the drink. After consuming the assigned drink, a blood sample will be collected 30, 60 and 90 minutes later, to determine leucine appearance (AUC). This procedure will be repeated three times, each with a different drink, with 5-7 days washout in between. The primary measure of the study is the leucine content in the blood, determined using High-Performance Liquid Chromatography (HPLC).
Control Intervention	Each participant acts as his/her own control. The usual inter-subject variability is minimized by the cross-over design as each participant is used as their internal control as each drink is tested in every participant.
Study procedures	- Recruitment and Screening (Month 1): Recruitment and screening aim to enroll 14 participants within one month. - Lab Visits (Months 2-3): Participants undergo three lab visits of about 4 hours at Laboratory Exercise and Health, ETH Zurich. Patients arrive fasted at the lab. Each visit includes a 60-minute seated rest, followed by one blood sample collection before and two blood sample collections after drink consumption. Samples are processed and stored at -80°C for further analysis. - Analysis (Month 4): High-Performance Liquid Chromatography (HPLC) used to measure leucine content in blood samples, assessing protein bioavailability.
Study Duration and Schedule	Planned 06/2024 of First-Participant-In Planned 09/2024 of Last-Participant-Out
Investigator(s)	Dr. Gommaar D'Hulst Laboratory of Exercise and Health Department of Health Sciences and Technology ETH Zürich Schorenstrasse 16, 8603 Schwerzenbach dhulstg@ethz.ch Tel: +41 78 662 44 74
Study Center(s)	Laboratory Exercise and Health, ETH Zurich, Schorenstrasse 16, 8603 Schwerzenbach
Data privacy	Privacy of data is guaranteed. Unique identifiers will be assigned to participants. This means that the data collected, analyzed, and reported cannot be traced back to individual participants. Secure Data Storage: All data, both physical and electronic, is stored in secure, access-controlled environments. All data is stored in an encrypted digital system (REDCap). Biological material in this study is not identified by participant name but by a unique participant number. Biological material is appropriately stored in a restricted area only accessible to the authorized personnel.

	Access to data will be restricted to authorized personnel, and appropriate encryption techniques will be applied to protect participant confidentiality.
Ethical consideration	The scientific value of this study lies in its evaluation of the bioavailability of leucine from a plant-based protein drink as compared to an animal-based one. This research is particularly relevant in the field of nutrition science, as it addresses the challenge of developing sustainable, plant-based protein sources that are nutritionally comparable to animal proteins. The study contributes significantly to the understanding of how plant-based diets can be optimized for enhanced human health, aligning with sustainability objectives in food production. Currently, there is a gap in knowledge regarding how a beverage specifically optimized for amino acid bioavailability, particularly leucine, stacks up against traditional animal-based protein drinks. This study aims to fill that gap, providing valuable insights into the potential of plant-based proteins in meeting nutritional needs. Justification of Methodology: High-Performance Liquid Chromatography (HPLC) has been selected for its accuracy in measuring specific amino acids, such as leucine, in blood samples. This method is known for its precision, making it ideal for quantifying amino acid levels accurately. The use of a randomized crossover design in this study ensures that each participant serves as their own control. This approach significantly enhances the reliability of the results, as it reduces the variability that can arise from individual differences between participants. Standard procedures like fasting and routine blood sampling are incorporated to accurately assess the bioavailability of nutrients. Fasting ensures that the baseline levels of amino acids are consistent across participants, while routine blood sampling allows for the tracking of changes in leucine levels over time following the consumption of the test drinks. The balance of risk and benefits for the participants, and by extension, for society, is carefully considered in this study. Participation is voluntary, and participants are expected to visit the lab three times over a short period of three weeks, each visit lasting approximately 4 hours. They will be compensated CHF 100 for their time and expenses. The study involves minimal invasive procedures, including the consumption of an animal- or plant-based drink and blood sampling. Considering these aspects, the study is structured to maintain a fair balance between risks and benefits, ensuring ethical integrity and participant safety while aiming to contribute valuable insights to the field of nutrition science.
GCP Statement	This study will be conducted in compliance with the protocol, the current version of the Declaration of Helsinki, the ICH-GCP, the HRA as well as other locally relevant legal and regulatory requirements.

2 BACKGROUND AND RATIONALE

Sufficient protein is very important for health, living well, and living longer (West et al., 2012). It is not just about how much protein a person eats, but also about its quality and where it is sourced. Proteins from animals are usually better because they are high in leucine and are easy to digest. However, they are not as good for the environment because farming animals on a large scale uses a lot of energy and water, and leads to more greenhouse gas emissions (Godfray et al., 2018). Addressing these environmental concerns, our team has developed a unique beverage from plant-based proteins, aimed at improving amino acid bioavailability. This is crucial, as low overall protein intake, combined with decreased bioavailability in plant proteins can lead to malnutrition, particularly in the elderly (Hida et al., 2014; van Vliet et al., 2015; Villet et al., 2005). Malnutrition in elderly is a huge societal burden costing Switzerland alone up to 500 Mio CHF per year due to longer hospitalization, increased risk of falling and muscle wasting (Frei, 2006). With an increasing demand for plant-based options among the aging population, our study will compare this new drink's nutritional and amino acid bioavailability with traditional animal-based products. Our goal is to provide a sustainable choice that maintains the nutritional advantages typically found in animal proteins.

The risk level of this study is considered low (A) because it only involves minor procedures like drawing blood and drinking a beverage that is safe for consumption. We think the advantages of this research far outweigh the slightly invasive methods, as it will significantly increase knowledge and interest for the benefit of society related to the effectiveness of plant-based drinks in tackling sarcopenia.

Scientific Value of the Study:

The study aims to evaluate the bioavailability of leucine from a plant-based protein drink compared to an animal-based protein drink (D'Hulst et al., 2021). Bioavailability is defined here as the appearance of amino acids in the blood plasma after consuming a protein-rich drink (Beal & Ortenzi, 2022). This has significant implications in nutrition science, especially for developing sustainable, plant-based protein sources that can match the nutritional value of animal proteins. Adequate levels of leucine in the blood and muscles are connected to increased muscle protein synthesis (D'Hulst et al., 2022), which over time helps in maintaining and growing muscles (Joanisse et al., 2020). Plant-based diets often have less leucine and lower bioavailability, which could lead to faster muscle loss if the protein content is not increased to levels usually found in animal-based diets (van Vliet et al., 2015). This project aims to explore how plant-based diets can be improved for better human health and to meet sustainability goals in food production. We have developed a drink that can be consumed by both older and younger people, containing a special blend of plant proteins that, according to our initial data, improves bioavailability and leucine absorption. However, it is still not clear how this optimized beverage compares to animal-based protein drinks, particularly in terms of leucine content. Initially, we aim to conduct a pilot study where younger (<40 y/o) participants are tested. Using that data and expertise, we aim to conduct a larger RCT in older patients once this pilot is finished. The purpose of this pilot study is to refine our experimental methods, choose the best drinks for testing in a larger trial involving older patients, and determine the right number of participants. We have set the upper age limit at 40 years to ensure that age-related factors don not interfere with our results on protein absorption.

3 STUDY OBJECTIVES AND DESIGN

3.1 Hypothesis and primary objective

Hypothesis:

We hypothesize that our plant-based, food-grade drink will result in blood leucine levels reaching up to 90% of those achieved by consuming an existing animal-based drink (Fresubin by Fresenius Kabi) or a comparable self-formulated animal-based drink. The self-formulated drink will contain the same ingredients as the plant-based one, except that the plant-based proteins will be replaced with animal-based whey protein.Primary objective: The objective of the study is to quantitatively assess the bioavailability of leucine in the blood following the consumption of a novel, but fully food-grade plant-based protein drink. This assessment will be compared to the bioavailability observed after consuming a animal-based protein drinks (commercial and self-formulated). By measuring leucine levels in the blood using High-Performance Liquid Chromatography (HPLC), the study aims to determine whether the plant-based drink can match or exceed the bioavailability of amino acids typically seen in animal protein sources, thereby offering a viable nutritional alternative for those seeking plant-based diets.

3.2 Primary endpoint

Primary endpoint: Leucine bioavailability in blood (HPLC), assessed by Area Under the Curve (AUC). We specifically chose leucine as this is the primary amino acids that triggers the synthesis of new proteins in the muscle via mechanistic target of rapamycin (D'Hulst et al., 2020). Typically, when a protein source is rich in leucine and consumption of it leads to a high level of leucine in the blood after consumption, like with whey protein, it promotes more muscle protein synthesis compared to a drink based on a single plant protein source, such as soy. Therefore, with our new

drink, we aim to optimize the appearance of leucine in the blood, which is a good indicator of protein synthesis in resting muscles. Age does affect bioavailability and protein synthesis (Schiaffino et al., 2013), hence, for this pilot trial we opt to study young, healthy individuals, males and females.

3.3 Study design

This will be an exploratory, single center, randomized, single-blinded, cross-over controlled pilot study with three arms: animal-based A, animal-based B, plant-based A. This study is designated as a 'pilot' because its objective is to generate scientifically valid data that can be leveraged for a more extensive future study focusing on older individuals experiencing malnutrition.

This trial design has the highest scientific validity as each participant will serve as their own control, and randomization determines the order in which the participants receive the drinks. The usual inter-subject variability is minimized by the cross-over design as each participant is used as their internal control as each drink is tested in every participant.

This study is a preliminary step (pilot) where we will examine how well leucine from various protein drinks is absorbed by people younger than 40 years old, as detailed in section 3.4. The findings from this initial study will help us design and carry out a more extensive, main study with older participants.

3.4. Study intervention

Participants will be administered three different drinks in a randomized crossover order. These drinks are:

- Animal-based A: A commercially available high protein drink, based on animal proteins (Fresubin Protein Energy Fresenius Kabi).
- Animal-based B: A homemade drink containing animal-based proteins, using a specific animal-based protein isolate mix.
- Plant-based A: A homemade drink containing plant-based proteins, formulated to have the same macronutrient content as the animal-based drinks.

** all drinks will have the same total protein content, and all participants will receive the same total amount of proteins.

Composition of drinks:

Animal Drink A:

https://www.fresenius-kabi.com/de-ch/produkte/fresubin-protein-energy-drink

https://www.fresubin.com/sites/default/files/2020-02/Productsheet_Fresubin%20Protein%20Energy_2019.pdf

Average content		100 ml	bottle = 200 ml
Energy value	kJ (kcal)	630 (150)	1260 (300)
Fat	g	6.7	13.4
of which SFA*	g	0.6	1.2
of which MUFA**	g	4.9	9.8
of which PUFA***	g	1.2	2.4
Carbohydrate	g	12.4/12.1[b]	24.8/24.2[b]
of which sugars	g	6.5[a]/6.9[c]/7.1[d]/7.4[b]	13[a]/13.8[c]/14.2[d]/14.8[b]
of which lactose	g	≤ 0.4	≤ 0.8
Fibre	g	0/0.5[b]	0/1.0[b]
Protein	g	10.0	20.0
Salt (Na x 2.5)	g	0.13/0.15[b]	0.26/0.30[b]
Water	ml	79	158
Osmolarity	mosmol/l	380/390[a]	
Minerals and trace elements			
Sodium	mg	50/60[a]	100/120[a]
Chloride	mg	73/58[a]	146/116[a]
Potassium	mg	130/135[a]	260/270[a]
Calcium	mg	205	410
Phosphorus	mg	120	240
Magnesium	mg	28/18[a]	56/36[a]
Iron	mg	2.5	5.0
Zinc	mg	2.0	4.0
Copper	µg	375	750
Iodine	µg	37.5	75.0
Selenium	µg	13.5	27
Manganese	mg	0.5	1.0
Chromium	µg	12.5	25.0
Molybdenum	µg	18.8	37.6
Fluoride	mg	0.25	0.5
Vitamins and other[e] substances			
Vitamin A	µg RE[o]	213	426
of which β-Carotene	µg RE[o]	63	126
Vitamin D_3	µg	2.5	5.0
Vitamin E	mg α-TE[oo]	3.75	7.5
Vitamin K_1	µg	21	42
Vitamin C	mg	18.8	37.6
Thiamin (vitamin B_1)	mg	0.3	0.6
Riboflavin (vitamin B_2)	mg	0.4	0.8
Vitamin B_6	mg	0.43	0.86
Niacin	mg/mg NE[ooo]	1.5 / 3.18	3.0 / 6.36
Folic Acid	µg	62.5	125.0
Vitamin B_{12}	µg	0.75	1.5
Pantothenic Acid	mg	1.5	3.0
Biotin	µg	9.4	18.8
Caffeine[e]	mg	0.65[a]	1.3[a]

Caloric distribution (energy%)
Fat 40, carbohydrate 33/32.3[a], fibre 0/0.7[a], protein 27

a = Flavour Cappuccino, b = Flavour Chocolate, c = Flavour Nut,
d = Flavour Tropical Fruits, e = Flavour Vanilla, f = Flavour Wild Strawberry
*saturated fatty acids (SFA), **monounsaturated fatty acids(MUFA),
***polyunsaturated fatty acids (PUFA)
°retinol equivalents (RE), °°alpha-tocopherol equivalents (α-TE), °°°niacin equivalents (NE)

Fresubin Protein Energy DRINK, flavour Wild Strawberry: Water, milk protein, vegetable oils (sunflower oil, rapeseed oil), maltodextrin, sucrose, flavourings, potassium citrate, beetroot powder, emulsifiers (E 471, soya lecithins), sodium chloride, vit. C, magnesium oxide, magnesium citrate, iron pyrophosphate, zinc sulphate, manganese chloride, vit. E, pantothenic acid, niacin, copper sulphate, vit. B_2, vit. B_6, sodium fluoride, vit. B_1, β-carotene, vit. A, folic acid, chromium chloride, potassium iodide, sodium molybdate, sodium selenite, vit. K_1, biotin, vit. D_3, vit. B_{12}.

Animal Drink B		
Reference Unit	Unit	Content per 100 ml
Energy, Calories	kcal	150
Fat	g	6.6
Carbohydrates	g	12.7
- of which sugars	g	8.8
Protein	g	10

- of which leucine	g	1
Plant Drink A		
Reference Unit	Unit	Content per 100 ml
Energy, Calories	kcal	150
Fat	g	6.9
Carbohydrates	g	11.7
- of which sugars	g	8.4
Protein	g	10
- of which leucine	g	0.8

Vitamin and mineral content of drinks (same for Animal Drink B and Plant Drink A):

Nutrient	Unit	Content per 100 ml
Vitamin A (µg-RE)	µg	213
Sodium (mg)	mg	50
Vitamin D (µg)	µg	2.5
Potassium (mg)	mg	130
Vitamin E (mg α-TE)	mg	3.75
Chloride (mg)	mg	73
Vitamin K (µg)	µg	21
Calcium (mg)	mg	205
Vitamin C (mg)	mg	18.8
Phosphorus (mg)	mg	120
Thiamin (Vitamin B1) (mg)	mg	0.3
Magnesium (mg)	mg	28
Riboflavin (Vitamin B2) (mg)	mg	0.4
Iron (mg)	mg	2.5
Niacin (Vitamin PP) (mg NE)	mg	1.5
Zinc (mg)	mg	2
Vitamin B6 (mg)	mg	0.43
Copper (mg)	mg	0.375
Folic Acid (µg)	µg	62.5
Manganese (mg)	mg	0.5
Vitamin B12 (µg)	µg	0.73
Selenium (µg)	µg	13.5
Biotin (µg)	µg	9.4
Chromium (µg)	µg	12.5
Pantothenic Acid (mg)	mg	1.5
Molybdenum (µg)	µg	18.8
Iodine (µg)	µg	37.5
Fluoride (mg)	mg	0.25

Drinks will be manufactured in a Food Grade Laboratory, LFO, ETH Zurich using standard mixing and homogenization procedures. Drinks will be sterilized using pasteurization (heating to 80 °C for 1 min. Exact composition of the drinks will be verified by Eurofins Scientific (https://www.eurofins.com/)

Participants will arrive fasted at the lab. After a 60 min seated rest, a baseline blood sample will be taken 30 minutes prior to consuming the drink. After consuming the assigned drink, a second, third and fourth blood sample will be collected 30, 60 and 90 minutes later, to determine leucine appearance (Area Under the Curve, AUC). This procedure will be repeated three times, each with a different drink, with 5-7 days washout in between. The primary measure of the study is the leucine content in the blood, determined using High-Performance Liquid Chromatography (HPLC), to assess the bioavailability of the proteins in the drinks.

We are testing two kinds of animal-based protein drinks and one plant-based protein drink. The reason for this is we are creating alternatives to the nutritional drinks currently sold by companies like Nestlé and Fresubin (FSMP products). We have made a drink using whey (which comes from animals) and another using a combination of pea and rice (which are plants), ensuring both have the same mix of nutrients as the ones you can buy now. We want to see if our drinks are better at delivering leucine to the body compared to the existing products.

4 STUDY POPULATION AND STUDY PROCEDURES

4.1 Inclusion and exclusion criteria, justification of study population

A total of 12 healthy volunteers will be recruited for this study of which 6 males and 6 females.

Inclusion criteria

- Gender: male and female
- Age: 18-40 years
- Signed written informed consent

Exclusion criteria

- Smoking history
- Cardiovascular diseases
- BMI > 35 kg/m2
- Caffeine consumption < 1 day before the study participation
- Allergies: Soy, Peas, Milk, Lactose, Fructose
- High intensity exercise <2 days before study participation

4.2 Recruitment, screening and informed consent procedure

The participant recruitment will occur using advertisements and social media within the Zurich area. The investigators will explain to each participant the nature of the study, its purpose, the procedures involved, the expected duration, the potential risks and benefits and any discomfort it may entail. Each participant will be informed that the participation in the study is voluntary and that he or she may withdraw from the study at any time and that withdrawal of consent will not have any consequences. All participants for the study will be provided a participant information sheet and a consent form describing the study and providing sufficient information for participant to make an informed decision about their participation in the study. One week time will be given to the participant to decide whether to participate or not. The formal consent of a participant, using the approved consent form, will be obtained before the participant is submitted to any study procedure.

The consent form will be signed and dated by the investigator or his designee at the same time as the participant sign. A copy of the signed informed consent will be given to the study participant. The consent form will be retained as part of the study records. After potential participants have given their informed consent, the project leader will accurately verify again the inclusion and exclusion criteria of the study. If all inclusion and exclusion criteria are fulfilled, participants will be included in the study. Study participants will receive CHF 100 for their participation into the study, after completion of the third experimental arm.

4.3 Study procedures

Overall Project Duration:

The project encompasses a total duration of four months, inclusive of the recruitment period and participation for each study subject. All the study procedures are summarized with a time schedule in Table 1.

Sequence of Planned Procedures:

1. **Recruitment and screening (Month 1)**

Recruitment and screening are projected to span one month, with the goal of enrolling 14 study participants.

- Participants will be recruited through advertisements and social media.
- Screening procedures will ensure eligibility criteria are met.

2. **Lab Visits (Months 2-3):**

Participants will undergo three lab visits at Laboratory Exercise and Health, D-HEST, Schorenstrasse 16, 8603 Schwerzenbach

- Participants will arrive fasted at the lab (no food and beverages starting at midnight)
- Each lab visit involves a 60-minute seated rest, followed by blood sample collection minutes before consuming the drink and 30, 60 and 90 minutes after consuming the assigned drink (see point 3. below).
- Three following three different drinks will be administered in a randomized order, with 5-7 days washout between visits:
    - Animal-based A: A commercially available high protein drink, based on animal proteins (Fresubin Protein Energy).
    - Animal-based B: A homemade drink containing animal-based proteins
    - Plant-based B: A homemade drink containing plant-based proteins, formulated to have the same macronutrient content as the animal-based drinks.

- All ingredients of the drink are food grade and prepared at a food-grade level (Laboratory of Sustainable Food Processing, ETH Zurich). Exact ingredients are: Plant protein isolates (pea, rice, hemp, pumpkin), Animal-based isolates (whey) water, maltodextrin, fruit puree (raspberry). All ingredients are commercially available.

- Total volume of the drink will range from 250ml to 400ml depending on the bodyweight of the participant

- During the first visit there will be also a short assessment of participants' characteristics (i.e. sociodemographic data and self-reported physical activity level). The following characteristics will be collected by the project leader/study personnel, inserted in a dedicated electronic Case Report Form (eCRF) within a web-based Electronic Data Capture (REDCap) system.

Procedure routine blood sample collection

Blood Sample Collection: Venous blood samples will be taken by a registered nurse during the 3 lab visits at four time points - 30 minutes before consumption of the drink, 30 minutes, 60 minutes and 90 minutes after the consumption of each drink. Blood will be drawn from the participant using a catheter. The selected vein, in the arm, will be disinfected with an alcohol swab. A catheter will then be inserted into the vein, and blood will be collected into appropriate tubes. After the blood collection, the catheter will be removed, and pressure will be applied to the puncture site with gauze or a cotton ball. The site will then be covered with an adhesive bandage or tape. All procedures will be performed following standard infection control protocols.

Throughout the whole protocol each day, approximately 30 ml of blood will be drawn (3 x 10 ml).

Processing and Storage: Blood will be centrifuged to separate plasma, and the samples are stored in -80 until further analysis.

3. Analysis (Month 4):

High-Performance Liquid Chromatography (HPLC): The primary measure of the study is the leucine content in the blood. This method ensures precise assessment of protein bioavailability in the administered drinks. This is a method used for separating, identifying, and quantifying each component in a mixture. In our study, HPLC will be used to analyze the leucine content in blood samples. It is a precise method that allows for the effective measurement of specific amino acids like leucine. We will only measure leucine from the blood.

Expected Biases and Mitigation Measures:

We acknowledge potential biases in the study, such as order effects and participant compliance. To mitigate these biases:

- Randomized order of drink administration minimizes order effects
- Drink administration is conducted in a single-blinded manner.
- Strict adherence to the washout period reduces carryover effects.
- Participants will receive thorough instructions and reminders to enhance compliance.

Table 1. Timeline of study procedures

Time (Months)	***Month 1***	***Month 2-3***			***Month 4***
Visit	*Information*	*Lab visit 1*	*Lab visit 2*	*Lab visit 3*	*Analysis*
Recruitment and screening	+				
Written consent	+				
Check inclusion-/ exclusion criteria	+				
Participant Characteristics		+			
Questionnaire		+			
Sampling		+	+	+	
Assessment of primary endpoints					+

4.4 Withdrawal and discontinuation

Participants will discontinue from the study in case of withdrawal of their informed consent or premature participant withdrawal (i.e. before Lab visit 3). In this case, the collected study-related

data and blood samples will be destroyed. After data analysis, study-related data will be anonymized, except when the concerned participant will expressly renounce to this right.

5 STATISTICS AND METHODOLOGY

5.1. Statistical analysis plan and sample size calculation

Our null hypothesis posits that the consumption of our plant-based, food-grade beverage does not result in a statistically significant difference in blood leucine levels compared to the consumption of an animal-based drink currently available in the market (Resource, Nestlé). Specifically, we anticipate that the leucine level attained by our plant-based drink does not exceed 90% of the level achieved by the animal-based alternative.

The study employs a crossover randomized controlled trial (RCT) design to investigate differences in leucine absorption, as measured by Area Under the Curve (AUC), following the consumption of animal drink A, animal drink B, and plant drink C. Each participant serves as their own control, receiving each of the three treatments in a randomized sequence over separate periods.

Detailed statistical plan:

1. Test for Normality

The Shapiro-Wilk test, with a significance level (α) set at 0.05, will assess the normality of the distribution of our variables. This test helps determine the appropriateness of parametric tests such as ANOVA for our data.

2. ANOVA

Our analysis will feature an ANOVA to examine the main effects of each drink (factors A, B, and C) and their interaction effects. This involves calculating sums of squares, degrees of freedom, mean squares, and F-statistics to understand each factor's impact and the interactions among them. We will interpret these effects, reporting the overall F-statistic and its associated p-value, and use a significance level to declare statistical significance.

3. Post-hoc Comparisons

Should the ANOVA indicate significant effects, post-hoc comparisons using methods such as Tukey's HSD or Bonferroni correction will pinpoint specific group differences. We will report adjusted p-values and confidence intervals for each comparison, providing detailed insight into the drinks' effects.

4. Effect Size

To test the practical significance of our findings, we will calculate effect sizes, such as beta-squared or partial beta-squared, for each factor and interaction. Power analysis

The power analysis was informed by a precedent human study (PMID: 36615694) that established average leucine AUC values to be 20,000 (with a standard deviation of 2,000) for plant-based drinks and 28,000 (with an updated standard deviation of 6,100) for animal-based drinks (whey). The mean difference between plant and animal drinks was identified as 8,000 units from this research. This study was performed in men and women, with no differences in leucine AUC between genders.

Given the crossover design's ability to significantly reduce between-subject variability by comparing treatments within the same individual, a within-subject correlation coefficient of 0.5

was assumed for the analysis. This assumption facilitated a more precise estimation of the required sample size, accounting for the intrinsic efficiency of the crossover approach in detecting treatment effects.

Adjusting for the crossover design, the power analysis indicated that approximately **9-10** participants would suffice to achieve the desired statistical power of 0.80 and an alpha level of 0.05. This sample size estimation directly reflects the enhanced efficiency and reduced variability afforded by the crossover design, as compared to a traditional parallel-group RCT, in discerning significant differences in leucine AUC among the dietary interventions examined.

We will recruit 12 participants to account for dropouts (10-20%).

CODE used in Python to calculate the n number:

```
from math import sqrt
from statsmodels.stats.power import FTestAnovaPower

# Updated standard deviation for the animal-based group
sd_animal_updated = 6100

# Given mean difference between plant and animal drinks
mean_diff = 8000

# Recalculate the effect size with the updated standard deviation
effect_size_updated = mean_diff / sd_animal_updated

# Calculate Cohen's f squared from the updated effect size (for ANOVA)
cohens_f_squared_updated = effect_size_updated**2 / (1 + effect_size_updated**2)
cohens_f_updated = sqrt(cohens_f_squared_updated)

# Setup for ANOVA power analysis
power_analysis = FTestAnovaPower()

# Number of groups (treatments)
num_groups = 3

# Desired power and alpha level
power = 0.8
alpha = 0.05

# Calculate required sample size per group with the updated effect size
sample_size_per_group_updated = power_analysis.solve_power(effect_size=cohens_f_updated,
                                        alpha=alpha,
                                        power=power,
                                        k_groups=num_groups)

# Assumption for typical within-subject correlation in crossover dietary intervention studies
within_subject_correlation = 0.5

# Adjusting the sample size estimate for the crossover design
n_parallel = sample_size_per_group_updated
k = 3
r = within_subject_correlation

# Adjusted sample size for crossover design
n_crossover = n_parallel / (1 + (k - 1) * r)
```

```
print(f"Adjusted sample size for crossover design: {n_crossover}")
```

5.2. Handling of missing data and drop-outs

Missing data will be handled by complete case analysis. If the participant withdraws prematurely or drops out (i.e. before Lab visit 3), they will be replaced by recruitment of new subjects to ensure an adequate number of participants in the study.

6 REGULATORY ASPECTS AND SAFETY

6.1 Local regulations / Declaration of Helsinki

This study is conducted in compliance with the protocol, the current version of the Declaration of Helsinki, the ICH-GCP, the HRA as well as other locally relevant legal and regulatory requirements.

6.2 (Serious) Adverse Events and notification of safety and protective measures

An Adverse Event (AE) is any untoward medical occurrence in a patient or a clinical investigation subject which does not necessarily have a causal relationship with the trial procedure. An AE can therefore be any unfavourable or unintended finding, symptom, or disease temporally associated with a trial procedure, whether or not related to it.

A Serious Adverse Event (SAE) (ClinO, Art. 63) is any untoward medical occurrence that

- Results in death or is life-threatening,
- Requires in-patient hospitalisation or prolongation of existing hospitalisation,
- Results in persistent or significant disability or incapacity, or
- Causes a congenital anomaly or birth defect

Both Investigator and Sponsor-Investigator make a causality assessment of the event to the trial intervention, (see table below based on the terms given in ICH E2A guidelines). Any event assessed as possibly, probably or definitely related is classified as related to the trial intervention.

Relationship	Description
Definitely	Temporal relationship Improvement after dechallenge* Recurrence after rechallenge (or other proof of drug cause)
Probably	Temporal relationship Improvement after dechallenge No other cause evident
Possibly	Temporal relationship Other cause possible
Unlikely	Any assessable reaction that does not fulfil the above conditions
Not related	Causal relationship can be ruled out
*Improvement after dechallenge only taken into consideration, if applicable to reaction	

Both Investigator and Sponsor-Investigator make a severity assessment of the event as mild, moderate or severe. Mild means the complication is tolerable, moderate means it interferes with daily activities and severe means it renders daily activities impossible.

Reporting of SAEs (see ClinO, Art. 63)

All SAEs are documented and reported immediately (within a maximum of 24 hours) to the Sponsor-Investigator of the study.

If it cannot be excluded that the SAE occurring in Switzerland is attributable to the intervention under investigation, the Investigator reports it to the Ethics Committee via BASEC within 15 days.

Follow up of (Serious) Adverse Events

Describe the follow up procedures of participants terminating the study with reported ongoing (S)AEs until resolution or stabilisation.

Notification of safety and protective measures (see ClinO, Art 62, b)

If immediate safety and protective measures have to be taken during the conduct of the study, the investigator notifies the Ethics committee of these measures, and of the circumstances necessitating them, within 7 days.

6.3 (Periodic) safety reporting

An annual safety report (ASR) is submitted once a year to the local Ethics Committee by the Investigator (ClinO, Art. 43 Abs 1).

6.4 Amendments

Substantial changes to the study setup and study organization, the protocol and relevant study documents are submitted to the Ethics Committee for approval before implementation. Under emergency circumstances, deviations from the protocol to protect the rights, safety and well-being of human subjects may proceed without prior approval of the Ethics Committee. Such deviations shall be documented and reported to the Ethics Committee as soon as possible. Substantial amendments are changes that affect the safety, health, rights and obligations of participants, changes in the protocol that affect study objective(s) or central research topic, changes of study site(s) or of study leader and sponsor (ClinO, Art. 29). A list of substantial changes is also available on www.swissethics.ch. A list of all non-substantial amendments will be submitted once a year to the competent EC together with the ASR.

6.5 Notification and reporting upon completion, discontinuation or interruption of the study

Upon regular study completion, the Ethics Committee is notified via BASEC within 90 days (ClinO, Art. 38). The Sponsor-Investigator may terminate the study prematurely according to certain circumstances, e.g.

- Ethical concerns,
- Insufficient participant recruitment,
- When the safety of the participants is doubtful or at risk (e.g. when the benefit-risk assessment is no longer positive),
- Early evidence of harm or benefit of the experimental intervention

Upon premature study termination or study interruption, the Ethics Committee is notified via BASEC within 15 days (ClinO, Art. 38).

Template concerning the notification of completion, discontinuation or interruption of the clinical trial for this is available on www.swissethics.ch.

All biological materials and health-related data are archived and encrypted in a coded format upon termination of data analysis for 10 years.

A final report is submitted to the Ethics Committee via BASEC within a year after completion or discontinuation of the study, unless a longer period is specified in the protocol (ClinO, Art. 38).

6.6 Insurance

In the event of study-related damage or injuries, the liability of the institution ETH Zürich provides compensation, except for claims that arise from misconduct or gross negligence.

7 FURTHER ASPECTS

7.1 Overall ethical considerations

The scientific value of this study lies in its evaluation of the bioavailability of leucine from a plant-based protein drink as compared to an animal-based one. This research is particularly relevant in the field of nutrition science, as it addresses the challenge of developing sustainable, plant-based protein sources that are nutritionally comparable to animal proteins. The study contributes significantly to the understanding of how plant-based diets can be optimized for enhanced human health, aligning with sustainability objectives in food production.

Our team has formulated a plant-based drink that, based on preliminary data and specific formulations using a variety of plant sources, is expected to increase the bioavailability of leucine compared to drinks made from a single plant protein source. This aspect is crucial as it opens the possibility of creating plant-based protein options that are not only environmentally sustainable but also nutritionally effective.

Currently, there is a gap in knowledge regarding how a beverage specifically optimized for amino acid bioavailability, particularly leucine, stacks up against traditional animal-based protein drinks. This study aims to fill that gap, providing valuable insights into the potential of plant-based proteins in meeting nutritional needs.

Justification of Methodology: High-Performance Liquid Chromatography (HPLC) has been selected for its accuracy in measuring specific amino acids, such as leucine, in blood samples. This method is known for its precision, making it ideal for quantifying amino acid levels accurately. The use of a randomized crossover design in this study ensures that each participant serves as their own control. This approach significantly enhances the reliability of the results, as it reduces the variability that can arise from individual differences between participants. Standard procedures like fasting and routine blood sampling are incorporated to accurately assess the bioavailability of nutrients. Fasting ensures that the baseline levels of amino acids are consistent across participants, while routine blood sampling allows for the tracking of changes in leucine levels over time following the consumption of the test drinks.

The balance of risk and benefits for the participants, and by extension, for society, is carefully considered in this study. Participation is voluntary, and participants are expected to visit the lab three times over a short period of three weeks, each visit lasting approximately 4 hours. They will be compensated CHF 100 for their time and expenses. The study involves minimal invasive procedures, including the consumption of an animal- or plant-based drink and blood sampling. Considering these aspects, the study is structured to maintain a fair balance between risks and

benefits, ensuring ethical integrity and participant safety while aiming to contribute valuable insights to the field of nutrition science.

7.2 Risk-benefit assessment

The assessments conducted in this research project are associated with minimal risk for the participants. Minor discomfort and risks associated with routine blood sampling. The volume of blood to be collected during each sampling is minimal, and the methodology adheres to established clinical standards to ensure participant safety. The primary risks associated with the procedure are limited to those commonly associated with routine venipuncture, such as temporary discomfort and bruising.

The beverages utilized in this study encompass both commercially available drinks, composed of widely recognized ingredients, and a specially formulated non-commercial beverage composed of food-grade products. It is important to highlight that all ingredients used in both types of beverages are common and widely consumed, thus not introducing novel or unknown risks. Despite the normalcy of the ingredients, we acknowledge the unique formulation, as well as specific processing of the non-commercial beverage. We have taken utmost care to select ingredients with minimal allergenic potential, ensuring participant safety. Detailed information about the composition of both types of beverages will be provided during the informed consent process, and any participant concerns or questions related to allergens will be promptly addressed.

The project acknowledges the risk of unauthorized data access and the unintended identification of project participants. To mitigate these risks, robust data security measures will be implemented. Access to sensitive information will be restricted to authorized personnel, and appropriate encryption techniques (unique identification code, section 8.3) will be applied to protect participant confidentiality.

While the results of this research project may not yield immediate benefits for the participants, the study carries significant value in advancing our knowledge in plant-based nutrition. The research aims to contribute to the broader understanding of plant-based nutrition, potentially uncovering valuable insights that may benefit individuals and public health in the long term. The study holds the promise of contributing to the development of nutritionally equivalent plant-based alternatives to animal proteins. This has the potential to influence future dietary choices and support sustainable and health-conscious dietary practices.

8 QUALITY CONTROL AND DATA PROTECTION

8.1 Quality measures

Data handling and protection is conducted according to the data protection law in Switzerland. The Digital Trial Intervention Platform (dTIP https://dtip.ethz.ch/) will monitor study conduction and data collection. Before the start of the study, the Principal Investigator will instruct and train the study personnel on how to correctly insert the data in the eCRF within the REDCap system, in close collaboration with dTIP. During the study, data checks and queries will be regularly performed for each individual case to ensure data quality and integrity. All study personnel agree to allow the Principal Investigator and dTIP the direct access to all relevant study documents. At the end of data collection, the Principal Investigator and dTIP will perform a final check for data quality and integrity and blood samples that will be used for analysis will be inserted into an eCRF within the REDCap System. All queries will be resolved before data analysis. To ensure proper blood sampling, an experienced nurse will be responsible for collecting blood samples. Subsequent to the collection, trained study personnel will handle the preparation of blood samples and perform High-Performance Liquid Chromatography (HPLC) analyses. For quality assurance the sponsor, the Ethics Committee or an independent trial monitor may visit the research sites.

Direct access to the source data and all study related files is granted on such occasions. All involved parties keep the participant data strictly confidential.

8.2 Data recording and source data

This ensures accountability, regulatory compliance, and data integrity throughout the study, participants' data will be collected and inserted by the project leader/study personnel into eCRF within the REDCap System. Project data resulting from the analyses of blood samples will be first inserted in an Excel file and stored in the ETH Server, and then transferred into an eCRF within the REDCap System. All data processing will be done locally and on password-secured computers of the ETH Zurich with a backup system. The REDcap System is implemented in the ETH server.

Project-specific source data:

- Patients' characteristics and questionnaire inserted in an eCRF within the REDCap System;
- Blood sample outcomes inserted in an Excel file stored in the ETH Server, and in an eCRF within the REDCap System.
- Data collected in paper Case Report Forms (p-CRF) stored in a lockable cabinet in the Laboratory Exercise and Health, ETH Zürich

REDCap maintains a built-in audit trail that logs all user activity and all pages viewed by every user, including contextual information (e.g. the project or record being accessed), entering data, exporting data, modifying a field, running a report, or add/modifying a use.

8.3 Confidentiality and coding

Trial and participant data will be handled with uttermost discretion and is only accessible to authorized personnel who require the data to fulfil their duties within the scope of the study. On the CRFs and other study specific documents, participants are only identified by a unique participant number. After recruitment, a study identification code will be generated for each participant. The study identification code is a unique alphanumerical code that prohibits the identification of the physical persons. All project-related data will be named using these identification codes. The code will be generated as a combination of letters and numbers based on (i) project name, (ii) participant number, (iii) year of birth (e.g., LEU011987). Health related personal data and biological samples of participants collected during the research project will all be named using these codes. Information about each participant identification code (e.g., name, address, date of birth) will be included in an Excel file, which is only accessible through name and password by the study personnel. The code will be broken if it is necessary to avert an immediate risk for the health of the concerned participant or to guarantee the rights of the participant (e.g., revoking the consent) or a legal basis exists for breaking the code. The coding will be done locally and on password-secured computers of the ETH Zurich with a backup system.

Biological material in this study is not identified by participant name but by a unique participant number. Biological material is appropriately stored in a restricted area only accessible to the authorised personnel. Biological material is appropriately stored in a restricted area only accessible to authorized personnel. Specifically, blood samples will be stored at -80°C (freezer is water cooled, key lock protected and is hooked up to a central alarm) in the Laboratory of Exercise and Health in Schwerzenbach. The study primarily focuses on analyzing blood parameters, specifically screening for leucine levels, utilizing non-genetic data.

8.4 Retention and destruction of study data and biological material

All study data and biological material are archived for 10 years after study termination or premature termination of the study at the Laboratory of Exercise and Health, ETH Zürich. After

10 years all study data will be deleted and biological material will be destroyed upon autoclaving.

9 MONITORING AND REGISTRATION

dTIP will oversee the monitoring of the clinical study. This external oversight ensures compliance with the approved protocol, ethical standards, and regulatory requirements. dTIP will closely monitor study procedures, data collection, and promptly addressing any deviations or safety concerns. Source data/documents are accessible to monitors and questions are answered during monitoring. The study is registered Swiss National Clinical trial Portal (SNCTP) and the addition of the study in the Clingov.org registry is ongoing currently.

10. FUNDING / PUBLICATION / DECLARATION OF INTEREST

The funding for the study is sourced from both ETH, Laboratory Exercise and Health and Innosuisse. The investigators will make every effort to present the research project results to national and international congresses, and - eventually - to publish the results of this pilot study in an international peer-reviewed journal. In terms of publication policy, the authors aim to adhere to recognized standards of academic integrity and transparency. They will ensure that appropriate authorship credit is given and that the raw data underlying the study findings are accessible to interested parties upon request. Timelines for publication may vary but will be pursued in a timely manner to facilitate the dissemination of results. Regarding data sharing policy, the investigators will uphold principles of data sharing where feasible and appropriate. They will consider requests for access to study data and collaborate with interested parties to facilitate meaningful analyses and interpretations. We will confirm that any observed gender effects will be disclosed in the final study report. The investigators declare no conflict of interest in planning, conducting and publishing the results of this study.

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