Strategies to Augment Ketosis: Optimization of Ketone Delivery(STAK:OK'd)

The Ohio State University

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Strategies to Augment Ketosis: Optimization of Ketone Delivery (STAK: OK'd) IRB Protocol

Title: Optimization of Ketone Delivery
Short title: STAK-OK'd

PI: Jeff S. Volek, PhD, RD
Professor, Department of Human Sciences
The Ohio State University
305 Annie & John Glenn Ave
Columbus, OH 43210
Office: 614-688-1701 | volek.1@osu.edu

Co-PI: Brianna Stubbs, DPhil
Lead Translational Scientist
The Buck Institue for Research on Aging
8001 Redwood Blvd
Novato, CA 94945
Office: 415-209-2000 | BStubbs@buckinstitute.org

ABBREVIATIONS

AcAc	Acetoacetate
BHB	Beta hydroxybutyrate
KE	Ketone ester
BDO	(R)-1,3 Butanediol
BTQ	Beverage tolerability questionnaire
LBM	Lean body mass
LC-MS	Liquid chromatography – mass spectrometry
VO_2	Oxygen uptake
VCO_2	Carbon dioxide release
GRAS	Generally recognized as safe
AUC	Area under the curve
C_{max}	Maximal concentration
C_{min}	Minimal concentration
T_{max}	Time of maximal concentration
T_{min}	Time of minimal concentration
mL	milliliters
mg	Milligrams
h	Hour
ANOVA	Analysis of variance
AE	Adverse event
USG	Urine Specific Gravity

STUDY SYNOPSIS

Study Title	Optimization of Ketone Delivery
Short Title	STAK-OK'd
Study Design	Open label, randomized, placebo controlled, 9-arm cross over
Study Participants	• Healthy men, aged 20 -30 years old, BMI between 18 and 29 kg/m^2
Planned Sample Size	An evaluable sample of 12 participants/group is required; to allow for 20% attrition a final sample size of 15 is planned.
Study Products	1. BHB Mono-ester 2. C6 Ketone Di-ester 3. AcAc Di-ester 4. (R)-1,3 butanediol 5. Control All study products are generally recognized as safe (GRAS) food ingredients.
Serving Sizes	1. 180 mg/kg of lean body mass 2. 360 mg/kg of lean body mass
Planned Study Period	September 2022 – September 2023
OUTCOMES	
Primary	Difference in total plasma ketone appearance (AUC) between the two serving sizes of study products and control.
Secondary	• Differences in other blood ketone kinetic parameters (peak concentration, time of peak concentration, AUC of different ketone bodies, capillary ketone concentrations). • Differences in urine and breath ketone excretion. • Differences in blood metabolites (glucose, free fatty acids, alanine). • Differences in blood hormones (insulin, ghrelin). • Differences in blood acid-base balance (bicarbonate, strong ions). • Differences in gas exchange (VO_2 and VCO_2). • Differences in heart rate and heart rate variability and blood pressure. • Differences in tolerability and subjective satiety.

SUMMARY AND BACKGROUND

This project is focused on nutrition-based STRATEGIES TO AUGMENT KETOSIS (STAK), which will enable people to increase blood ketone concentrations, achieve 'nutritional ketosis' which may possibly have beneficial effects on resilience and health. Ketone esters (KEs) represent one promising STAK method, but even within this category of exogenous ketones there are multiple compounds that vary in composition of ketone-promoting components joined by ester bonds. The ketone-promoting components can be ketone bodies such as beta hydroxybutyrate (BHB) or acetoacetate (AcAc), ketogenic precursors that metabolize to ketones via the classical beta-oxidation ketogenesis pathway (i.e., medium chain fatty acids) or ketone precursors that metabolize to BHB via a non-classical pathway (i.e., (R) -1,3 butanediol, **BDO**). Three Generally Recognized as Safe (GRAS) KE compounds exist, each with distinct pharmacokinetic and pharmacodynamic effects. Most recent clinical KE research has used a monoester of BHB and (R)-1,3 butanediol (**BHB Mono-ester**), with two more recently available compounds including a diester of hexanoic acid (a ketogenic medium chain fatty acid) and (R)-1,3 butanediol (**C6 Di-ester**) and a diester of AcAc and (R,S)-1,3 butanediol (**AcAc Di-ester**)(**Fig 1 and Table 1**). Whilst blood BHB kinetics of the BHB Monoester are well characterized at rest and during exercise[1-11], human studies of the two other KE compounds are limited. Recent studies of KEs suggest that they may have many acute functional effects, such as modulation of glucose, fatty acid and amino acid metabolism[5,6], heart rate[4], acid base homeostasis[7,8,11,12] and urine output[7,8], as well as multiple other effects demonstrated in preclinical research, including modulation of oxidative stress and inflammation[13,14], that are yet to be investigated in clinical studies. As the number and sophistication of KE products grows, there is a critical need to expand our knowledge of the similarities and differences between these compounds and how their metabolism varies between individuals to optimize dosing strategies for service members . Our long- term goal is to create evidence-based guidelines for deployment of diverse KE based on knowledge of each compound, the effect of individual phenotype, and operational functional requirements.

KE Metabolic Effects Vary Between Compounds. One important difference between KE compounds is the ketone-promoting components, which determines the circulating ratio of blood ketone bodies, BHB and AcAc, and may in turn lead to important metabolic and signaling differences. Whereas some actions of the ketone bodies BHB and AcAc are shared, R-BHB has a broad range of signaling functions that are distinct from AcAc, some of which are shared by the non-circulating, non-oxidizable enantiomer, S-BHB[13,14]. AcAc also has metabolic and signaling actions that are independent of BHB[15-18] and is selectively oxidized in some cells that cannot oxidize BHB[42]. Furthermore, responses to different ketone bodies vary between tissue types[15]. A second difference between KE arises from the balance between direct delivery of ketones compared to indirectly elevating ketone concentration via metabolism of non-classical or classical ketogenic precursors (**Figure 1**). Classical ketogenesis itself may drive adaptation and some of the functional benefits associated with ketosis[19]. BDO is included in all of the KE compounds, but it is currently

unknown how consumption of BDO alone, and its metabolism via non-classical ketogenesis acutely affects metabolism. Additionally, ketogenesis is now understood to occur in certain cells outside the liver with important local biological effects, for example ketogenesis driven by medium chain fatty acids has been reported in astrocytes *in vitro*[10]. Provision of systemic BHB by a KE may elicit different biological effects in some tissues such as the brain versus promoting *in situ* ketogenesis in that tissue. Overall, not only are functional effects of KE incompletely defined, but also it is unknown which effects are common to all KE versus which are specific to an individual KE compound (i.e., BHB Monoester vs AcAc Diester) or which may be attributable to the BDO precursor common to all of the KE.

This study will be the first comparative full crossover study of all available KE and the precursor BDO at two serving sizes. Outcomes will focus on established effects of the BHB Monoester (including the effects on ketones, glucose and acid-base balance) and compare these with the effects of the AcAc Diester, C6 Diester and BDO. The results of this project will provide information to facilitate selection of exogenous ketones based on the specific physiological and metabolic effects that they induce.

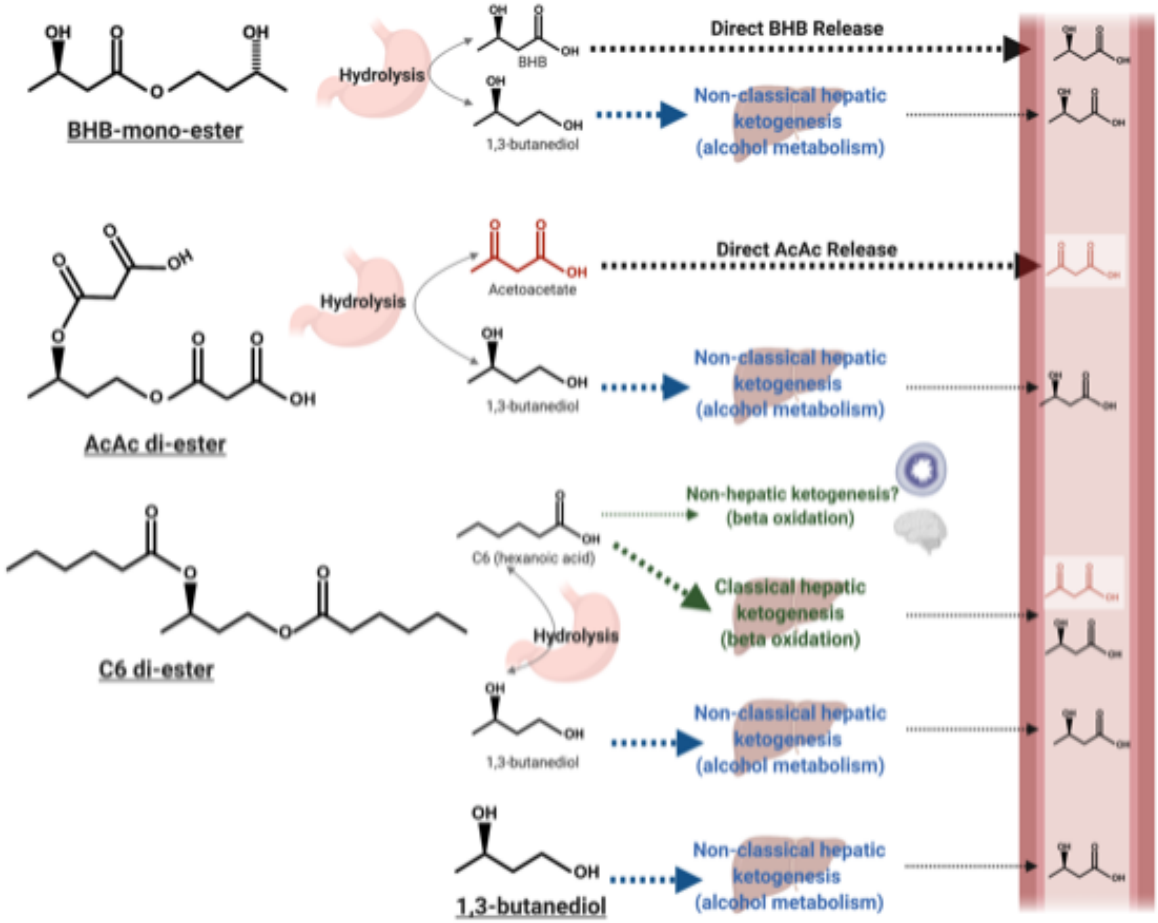

Figure 1| Metabolism of 3 ketone ester compounds and the BDO ketone-precursor.

IMPACT

KEs could represent a practical STAK method that does not require any change in diet. However, there is a critical need to elucidate unique attributes of the many new exogenous ketone compounds and how their metabolism varies between individuals to inform KE use in the field. This high impact project will contribute important knowledge that aims to develop next-generation KE molecules and formulations that are designed to meet the needs of operators at different points in their career. Our long-term goal is to create evidence-based guidelines for deployment of diverse KE compounds, dose level, and timing based on individual characteristics and operational/training requirements.

RESEARCH STRATEGY

This is an open label, randomized, placebo controlled, 9-arm crossover study where healthy adult men (n = 15) will consume four different exogenous ketone products at two serving sizes or a non-ketogenic placebo (one study product per test day). Data/sample collection will include blood (capillary and whole venous blood), gas exchange measures, urine, heart rate/variability and study product tolerability.

OUTCOMES

Primary

- Difference in total plasma ketone appearance (AUC) between the two serving sizes of study products and control

Secondary*

- Differences in other blood ketone kinetic parameters (peak concentration, time of peak concentration, AUC of different ketone bodies, capillary ketone concentrations).
- Differences in urine and breath ketone excretion
- Differences in blood metabolites (glucose, free fatty acids, alanine).
- Differences in heart rate and heart rate variability and blood pressure
- Differences in subjective tolerability and subjective satiety measured by questionnaires
- Differences in blood hormones (insulin, ghrelin).
- Differences in blood acid-base balance (bicarbonate, strong ions
- Differences in respiratory gas exchange (VO_2 and VCO_2

*All comparisons will be made between the two serving sizes of one study product and vs. control.

PARTICIPANTS

This study aims to recruit a homogenous, military relevant population to remove the confounding effects of variation in phenotype on exogenous ketone metabolism as far as possible. Thus, we will 15 recruit healthy and normally-active resistance/endurance trained men from the ages of 20-30 yr that represents typical active-duty military

personnel. Each participant must meet all of the inclusion criteria and none of the exclusion criteria at screening in order to participate

Inclusion criteria:

- Male
- BMI between 18 and 29 kg/m2
- Aged 20 – 30 years
- Participant is willing and able to comply with all study procedures including the following prior to Test Days: fasting (>10 h; water only), no alcohol (>24 h), no exercise (>24 h), no acute illness and controlled feeding before each Test Day, maintain diet, exercise, medication, and supplement habits throughout the study.
- Participant has no health conditions that would prevent completion of the study requirements as judged by the Investigator based on health history.
- Participant understands the study procedures and signs forms providing informed consent to participate in the study and authorizes the release of relevant protected health information to the Investigator.

Exclusion criteria:

- Participant follows a low-carbohydrate diet (<30% energy from carbohydrate) or have used exogenous ketone supplements within 4-months of study participation.
- Participant has a Primary Care Physician diagnosed history or presence of uncontrolled and/or clinically important hypertension (blood pressure >150/95 mmHg), pulmonary, cardiac, hepatic, renal, endocrine (including type 1 and 2 diabetes), hematologic, immunologic, neurologic (e.g., Alzheimer's or Parkinson's diseases), psychiatric (including unstable depression and/or anxiety disorders) or biliary disorders.
- Participant has a known allergy, intolerance, or sensitivity to any of the ingredients in the study beverages, including soy and milk protein, wheat, shellfish, fin fish, eggs, tree nuts or peanuts (production facility handles nuts).
- Participant has unstable use of a medication or supplement that the Investigator considers may affect the outcomes of the trial.
- Consumption of alcohol more than 3 drinks per day or more than 18 drinks per week.
- Consumption of tobacco.
- Consumption of cannabis.
- Participant is currently in another research study or has been in the 14 days before screening.
- Participant has had a blood draw or donation in the last 8 weeks.
- Participant has a clinically important gastrointestinal (GI) condition that would potentially interfere with the evaluation of the study beverage [e.g., inflammatory

bowel disease, irritable bowel syndrome, chronic constipation, severe constipation (in the opinion of the Investigator), history of frequent diarrhea, history of surgery for weight loss, gastroparesis, systemic disease that might affect gut motility according to the Investigator, medication managed reflux and/or clinically important lactose intolerance].

- Participant has a condition the Investigator believes would interfere with his ability to provide informed consent, comply with the study protocol, which might confound the interpretation of the study results, or put the participant at undue risk.

Sample Size. Our primary outcome goal is to demonstrate significant differences between serving sizes of exogenous ketone compounds in plasma total ketone AUC. Previous work using the BHB Monoester compared to ketone salt products demonstrated that a sample size of 12 participants was required to detect a difference in BHB AUC of ~20% (80 $mM.h^{-1}$) between ketone compounds with alpha set at 0.5 and power at 0.8. This sample size will also give sufficient power to address our secondary aims which include differences in peak ketone concentrations, plasma BHB, AcAc, breath acetone, glucose, insulin, free-fatty acids, amino acids, blood chemistry, pH, gas exchange values and heart rate. Based upon prior studies, we expect up to 20% of participants will withdraw from the study after recruitment, and therefore we will plan to recruit 15 participants.

Recruitment:
Recruitment will occur through well-established methods at OSU, which include word of mouth, ResearchMatch database, social media engagement and flyers. For this study we will specifically aim to recruit cadets in the OSU Reserve Officers Training Corps (ROTC), who have participated in research on ketosis in the past and been supported by their cadre to enroll in our research projects (see letters of support from Army, Navy, and Air Force ROTC leadership).

Randomization:
If a participant meets all inclusion and none of the exclusion criteria, the following will occur at the end of the screening visit:

- The randomization sequence will be prepared using an online random sequence generator (randomization.com) that will generate a list of random number sequences between 1 – 9 that correspond to product/serving code. If a participant meets all inclusion and none of the exclusion, a staff member will randomize the participant – in the order that they are enrolled. The randomization number will be recorded in the participant's source documentation.

METHODS

Study overview:

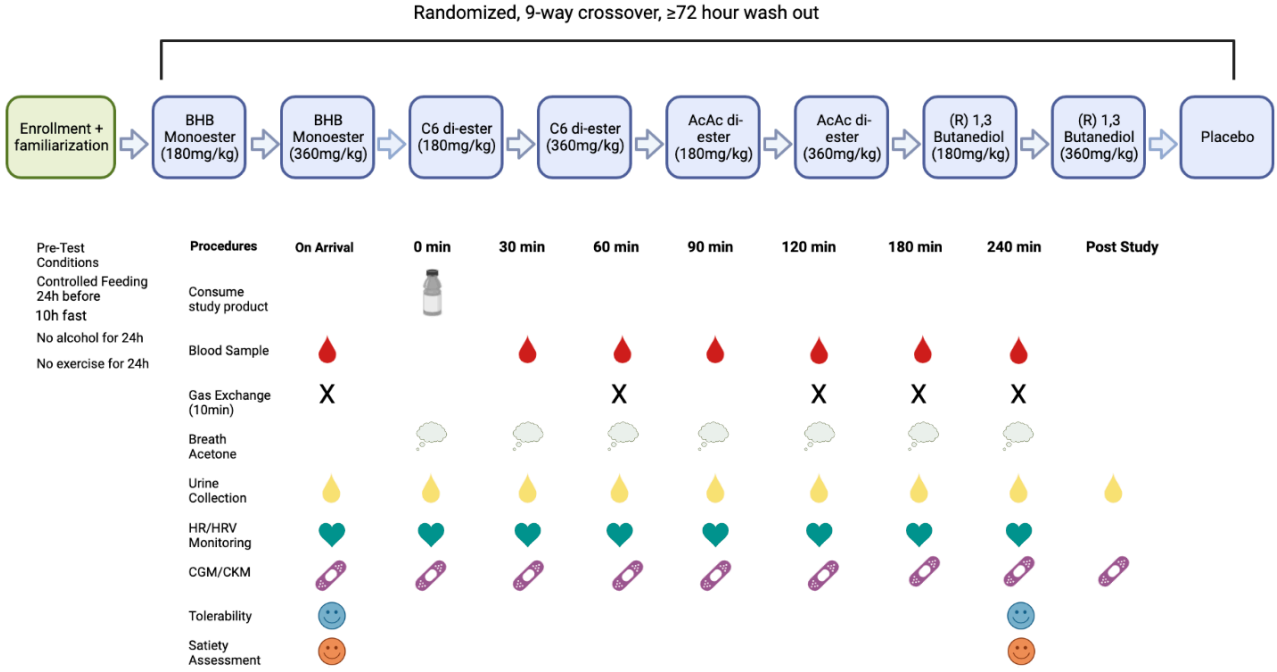

Figure 2 | Study schematic, showing timeline for a study day.

Study Procedures

Study Day	Screening	Test Days 1-9
Visit to Study Center	X	X
Informed consent	X	
Medical intake questionnaire, taste screening, diet assessment, anthropometrics[1]	X	
Randomization[2]	X	
Compliance assessment[3]		X
Consume study product[4]		X
IV Cannulation and whole blood collection[5]		X
Urine collection[6]		X
Respiratory gas analysis[7]		X
Heart rate and HRV monitoring[8]		X
Tolerability assessment[9]		X
Satiety assessment[10]		X
Continuous Ketone Monitor placement/check[11]		X

1. Medical intake questionnaire, taste screening diet assessment questionnaire and anthropometric data will include: Lean Body Mass by DXA, BMI, height and weight.
2. Order of study products will be randomized using a computer randomization generator.
3. Prior to starting each test day, you will be asked to confirm that you meet pre-test criteria, including: fasted >10h, no alcohol for 24h, no exercise for 24h and consumed the provided pre-test food.

4. Study Product will be consumed within +/- 60 minutes of the time established on the first test day. Participants will be provided with a choice of non-caffeinated, non-caloric beverage to remove the bitter taste of the Study Product.
5. IV cannula will be inserted at the start of each Test Day, and removed at the end of each Test Day, Blood samples will be collected according to the schedule in Figure 1. Cannula will be flushed with a small volume of saline after each sample to maintain patency. We will draw 504 mL of blood, which is about 2.1 cups throughout the 4-week intervention.
6. Prior to consumption of the Study Product, participants will be asked to completely void your bladder. And hydration status will be determined via urine specific gravity (USG) reporting <1.025. Urine passed after the ingestion of the study product will be collected in a plastic container; you will be asked to void their bladder and collect urine at the end of the test day. The volume produced will be recorded at the end of the study and aliquots will be frozen and stored for future analysis.
7. Participants will breathe into a commercially available handheld breath acetone analyzer according to the schedule in Figure 2. Participants will wear a fitted face mask attached to a metabolic cart for a 10-minute period every 60 minutes.
8. Participants will wear a Bluetooth heart rate monitor chest strap throughout the test day.
9. Participants will complete a Beverage Tolerability Questionnaire prior to Study Product consumption and at the end of the Test Day.
10. Participants will complete a 3-item satiety visual analogue scale prior to Study Product consumption and at the end of the Test Day.
11. Continuous Ketone Monitor will be applied at the start of Test Day 1. The sensor will be checked by the study team at each test day and will be removed and replaced by a fresh sensor at ~2- week intervals during the study. The sensor will be removed at the end of the final test day.

Screening Visit: Participants that meet the initial qualifying criteria will visit the study center for a screening meeting. The participant and a member of the research team will meet in a private office to discuss the informed consent form. The informed consent form will be provided to the participant for their review, the study will be described in full detail and any questions the interested participant has will be encouraged and responded to. If they choose to participate in the study, they will be asked to sign the consent form providing written consent. The participant will be informed that even though they signed the consent form, their participation in the study is dependent on anthropometric measures and diet and medical questionnaire answers to determine if they meet the study criteria.

If the participant provides consent, they will be provided with questionnaires including Automated Self-administered 24-hour Dietary Assessment Tool (ASA24®),and medical history. All collected samples and data will be coded to maintain participant anonymity. We will give the participants a small volume of Study Product to screen for tolerance of the bitter tasting Study Products. We will also measure height, weight and body composition using a DXA scanner. Their enrollment for the intervention will be determined from the results. If the participant is eligible for the study and is still interested in participating then they will be randomized to a study product order and scheduled to return to the study center for the first testing visit.

Testing Days:

Participants will report to the study center in the morning of each Test Day. Compliance with pre-test instructions (fasted > 10h, no alcohol >24h, no exercise >24h, consumed pre-test food) will be confirmed by the Investigator. Participants will complete a baseline Beverage Tolerability Questionnaire (BTQ) and satiety visual analogue scale. Participants will be asked to completely void their bladder and a sample will be analyzed for hydration status. Participants with samples reading greater ≥ 1.025 USG, and asked to don the Bluetooth heart rate monitor chest strap. A study team member will assist the participant with application of a continuous ketone meter into the back of the arm, this will be removed and replaced with a fresh sensor at Test Days at ~2-week intervals; the sensor will be removed 24 hours after the cessation of the last in lab testing bout. Participants will be given written instructions on how to remove and dispose of monitor. A trained member of the study team will insert an IV cannula into a vein in the antecubital fossa to allow for repeated blood sampling. The cannula will be flushed with a small volume of saline after each sample withdrawal to maintain patency. At the same time as all whole blood samples, we will also collect capillary blood samples from a finger for real-time analysis of blood BHB and glucose concentration, using lancing device, commercially available test strips and a handheld monitor (KetoMojo, CA, USA). Participants will wear a fitted facemask connected to a metabolic cart for 10 minutes to collect measures of respiratory gas exchange. Participants will exhale once into a commercially available handheld breath acetone analyzer (Readout, MI, USA). Baseline blood sample, baseline respiratory gas measures and baseline breath acetone will be collected.

Participants will then consume the Study Product that they were randomly allocated for that Test Day (details of Study Products below). Time of ingestion should be +/- 60 minutes from the time established at Test Day 1; they will be given 5 minutes to consume the Product. After Study Product consumption, they will remain at the study center for ~4h, with blood and breath sampling occurring at regular intervals (see Figure 2). A total of 7 whole blood samples (7x ~8 mL each= ~56mL) will be collected each Test Day. Capillary blood samples and breath acetone readings will be collected at the same time as whole blood samples. The fitted face mask will be worn for 10 minutes each hour to collect respiratory gas measures. Participants will be asked to collect all urine passed during the test in a provided container, and asked to fully void their bladder and collect the urine at the end of the test day. Participants will complete satiety questionnaires each hour, and will complete a BTQ at the end of the Test Day.

Participants will be asked to minimize ambulatory movement during the Test Day. Non caloric beverages (i.e.,water) will be permitted *ad libitum* and intake volumes will be recorded. At the end of each Test Day, the heart rate monitor and IV cannula will be

removed and a dressing will be applied to the cannula site. Participants will be given a snack to consume.

Sample Processing and Analysis

Blood Processing: Whole blood collected through the IV cannula will be either 1) immediately stabilized for AcAc analysis using a validated method, 2) processed to plasma (EDTA tubes), or 3) serum (clot activator serum collection tubes) and then snap frozen in liquid nitrogen for storage prior to analysis (details below). We will collect capillary blood samples for real-time analysis of blood BHB and glucose concentration, using commercially available test strips and a handheld monitor (KetoMojo, CA, USA), this will also allow comparison of ketone values obtained via different methods.

Blood Sample Analytical Methods:

- *Ketones* will be determined by multiple methods to provide a robust measure of ketosis and allow cross validation. Firstly: by capillary BHB concentrations obtained from a finger stick using a handheld device (KetoMojo, USA). Secondly: plasma ketones (AcAc, R-BHB and S-BHB) will be measured using ultra-performance liquid chromatography – tandem mass spectrometry (LC-MS). Thirdly: Continuous Ketone Monitoring (see details below)
- *Plasma hormone* (insulin, GIP, ghrelin) will be analyzed using commercially available ELISA assay kits (Cayman Chemical, USA).
- *Plasma metabolites* (free fatty acids, alanine, lactate) will be determined using standard enzymatic assays, which have been extensively used at OSU.
- *Whole blood clinical chemistry* will be performed using a commercially available, clinical grade handheld analyzer (iSTAT, Abott, USA) and cartridges which will deliver the following data: Sodium, Potassium, pH, PCO_2, Urea Nitrogen, Glucose, Hematocrit, TCO_2, HCO_3, Base Excess, Anion Gap, Hemoglobin (EC8+, Abott, USA).

Continuous Ketone Monitoring: We plan to incorporate the use of a novel continuous ketone monitoring (CKM) system developed by Abbott Diabetes Care. The ketone sensor in this CKM device is similar to the FreeStyle Libre continuous glucose monitoring (CGM) described previously[25], but it has been modified using wired enzyme technology with BHB dehydrogenase chemistry. The sensor adheres to the back of the arm where it continuously samples interstitial fluid for quantification of BHB concentration. The sensor is worn for a period of 2-wk, three sensors will be used to cover all test days in this study (6 weeks). The first sensor will be inserted with assistance from the study team at the start of Testy Day 1, it will be checked every visit and replaced at Test Days following ~2-week intervals. The sensor will be removed 24 hours after the cessation of the last in lab testing bout (Testing Day 9). Participants will be given written instructions on how to

remove and dispose of monitor. Feasibility, stability and other quality control parameters of this CKM has been established[26].

Urine Participants will void their bladder on arrival and hydration status will be determined via urine specific gravity (USG) reporting <1.025 and all urine passed during the study will be collected; volume will be recorded. Water intake (permitted *ad libitum)* will be recorded. Urine ketone concentration will be measured using a modified version of the LC-MS method described above.

Respiratory Gases: Respiratory gas exchange will be assessed using breath-by-breath gas exchange measurements of VO_2 and VCO_2 recorded every 30-sec.Participants will wear a fitted face mask connected to a metabolic cart for 10 minutes every hour (TrueOne 2400, ParvoMedics, UT, USA). Participants will also complete one exhale into a high-resolution portable breath acetone meter (Readout Health, USA) for determination of breath acetone.

Heart rate and heart rate variability: At the start of each Test Day participants will don a Bluetooth chest strap that is sensitive enough to detect R-R intervals (Polar Electro, Finland). The chest strap will be worn for the duration of each Test Day and removed before the participant leaves the study center.

Beverage Tolerability Questionnaire: The BTQ used in this study is similar to that used in previous tolerability studies[27,28,29,30] . Ten tolerability issues are included in the BTQ: gas/flatulence, nausea, vomiting, abdominal cramping, stomach rumbling, burping, reflux (heartburn), diarrhea, headache, and dizziness. Participants are asked if the issue was present (pre- beverage - baseline) or had occurred since they took the study beverage (post-beverage – 4h) at the following intensities: none, mild (awareness of symptoms but easily tolerated), moderate (discomfort enough to interfere with but not prevent daily activity) or severe (unable to perform usual activity). These correspond to scores of 0–3, respectively for each issue, giving a maximal composite score, defined as the sum of the ten items, of 30.

Satiety Visual Analogue Scale: We will use a 3-item visual analogue scale, that assesses hunger, fullness and desire to eat by participant's marking on a line anchored at either end with 'not at all' and 'extremely.' Distance along the line is measured in mm.

STUDY PRODUCTS

Background: The four exogenous ketone compounds (BHB Monoester, C6 Diester, AcAc Diester, (R) 1,3 Butanediol) will be used all have GRAS status as a dietary ingredient (**Table 1**). BHB Monoester, C6 Diester, and (R) 1,3 Butanediol are commercially available in the products 'deltaG' (TdeltaS Global, FL, USA), 'Metabolic Switch' (Juvenescence Ltd,

NJ, USA) and 'Avela' (Genomatica, CA, USA), respectively. The AcAc Diester is commercially available (on Amazon https://www.amazon.com/KetoLogic-Ketone-DiEster-Softgels) and is currently being used in clinical studies of Angelman's Disease[21]. We have been in communication with the companies that produce these exogenous ketones to ensure product availability and support for this work (see letters of support from BHB Therapeutics/Juvenescence and Disruptive Nutrition).

Quality Control: As only small amounts of product are needed for this study, and products are shelf stable, our aim is that all product inventory will be acquired at the start of the study to ensure the same product lots are used throughout. We plan to obtain Certificates of Analysis to confirm product exogenous ketone content.

Serving Size Rationale: Previous work has shown that lean body mass (LBM) is a major covariate in KE responsiveness[9]. Therefore, we will standardize exogenous ketone serving sizes to LBM (assessed using DXA) for all trials at 180 and 360 mg/kg LBM, which for a participant with 70 kg of lean mass corresponds to ~12.5 and 25 g, respectively. These serving sizes are representative of typical commercial serving sizes and are expected to elevate blood BHB in the range of 1.5 - 2 mM[22,23].

Blinding: It will not be possible to completely blind study participants or investigators due to visible differences between the C6 Ketone Di-ester and BHB-Mono-ester beverages and also the capsule delivery form for the AcAc Di-ester. However, study products will be coded (A, B, C, D and CON), and data analysts will remain blinded to the coding until analysis is complete.

Storage: The study products will be stored in a dry secure location at ambient temperature (15- 77°F) and are best served chilled. Study product supplies are to be used only in accordance with this protocol and under the supervision of the Investigator.

Table 1: Study product details

Study Active Ingredient	Serving Sizes	Form	Other product ingredients [ALLERGENS]	Example Commercial Product (Company)
BHB Mono-ester	180 mg/kg and 360 mg/kg	Ready to drink beverage	Water	DeltaG Tactical (TDeltaS Global, FL, USA)

C6 Di-ester	180 mg/kg and 360 mg/kg	Ready to drink beverage	Water, whey protein concentrate, modified gum acacia, natural and artificial flavors, cocoa powder, lecithin, fruit and vegetable juice for color, bisulfate of soda, caramel color, acesulfame K, cellulose gum, pectin (standardized with sucrose), sucralose, xanthan gum, canola oil [DAIRY AND SOY]	Metabolic Switch (Juvenescence Ltd, NJ, USA)
AcAc Di-ester	180 mg/kg and 360 mg/kg	1.5g capsules	Geltain (bovine), glycerin, water.	KetoLogic Ketone Di-ester Softgels (Disruptive Nutrition LLC)
(R)-1,3 butanediol	180 mg/kg and 360 mg/kg	Undiluted liquid ingredient – mixed on site with water and sweetener	None	Ketone-IQ (H.V.M.N. Inc, FL, USA)
Control	NA	Beverage	Water, canola oil, whey protein concentrate, modified gum acacia, natural and artificial flavors, cocoa powder, lecithin, fruit and vegetable juice for color, bisulfate of soda, caramel color, acesulfame K, cellulose gum, pectin (standardized with sucrose), sucralose, xanthan gum, canola oil [DAIRY AND SOY]	National Food Laboratory, NY, USA

DATA ANALYSIS AND STATISTICAL METHODS

Primary outcomes

- Difference in total plasma ketone appearance (AUC) between the two serving sizes of study products and control

Secondary outcomes

- Differences in other blood ketone kinetic parameters (peak concentration, time of peak concentration, AUC of different ketone bodies, capillary ketone concentrations).
- Differences in urine and breath ketone excretion
- Differences in blood metabolites (glucose, free fatty acids, alanine).
- Differences in heart rate and heart rate variability and blood pressure
- Differences in subjective tolerability and subjective satiety measured by questionnaires
- Differences in blood hormones (insulin, ghrelin).
- Differences in blood acid-base balance (bicarbonate, strong ions)
- Differences in respiratory gas exchange (VO_2 and VCO_2)

Statistical Plan. Statistical analysis of data will take place on completion of the study in collaboration with Buck Bioinformatics Core. Participant data will be included if they have completed at least 2 serving sizes of one exogenous ketone product AND the control Test Days (i.e., BHB- mono-ester 180 mg/kg, BHB mono-ester 360 mg/kg and Control). Any incomplete exogenous ketone compounds (i.e., participant completed C6 Di-ester 180 m/kg and AcAc ester at 360 mg/kg) will be excluded from the analysis. All decisions regarding exclusion from the analysis population will be documented prior to database lock. Missing data will not be imputed, and only observed data will be included in the statistical analysis.

Repeated measure group x time ANOVA (or a non-parametric alternative) will be performed following initial tests to ensure sphericity is not violated. We will compare data between control group vs. each individual exogenous ketone at both doses (i.e., control vs BHB-mono ester (180 mg/kg) vs BHB-mono-ester (360 mg/kg). We will also test for condition x time interactions. If significance is detected, post-hoc comparisons will be made using Tukey's HSD tests (or non-parametric alternative) to correct for multiple comparisons. Significance will be accepted at an alpha level of $P < 0.05$.

Other data processing:

- Area Under the Curve (AUC) will be calculated using the Trapezoid Rule.
- Maximal/minimal concentration data (C_{max} and C_{min}) and time of maximal/minimal concentration (T_{max} and T_{min}) will be extracted from the raw data.

- A composite tolerability score is calculated by adding the scores (0 -3, corresponding to none, mild, moderate, severe) of all ten listed symptoms.

STUDY MONITOTRING

Concomitant Medication/Supplements and Treatment

All concomitant medications/supplements used 1 months prior to Screening Visit and during the study will be reported to the study personnel for assessment and recorded in the participant's study documents.

Adverse Event Monitoring

An AE is defined as any untoward medical occurrence in an investigation participant following written informed consent that does not necessarily have a causal relationship with the study product. An AE can be any unfavorable or unintended sign (including an abnormal finding), symptom, or disease temporally associated with the use of an investigational product, whether or not related to the investigational product. This includes any occurrence that is new in onset or aggravated in severity or frequency from the baseline condition, or abnormal results of diagnostic procedures (including laboratory test abnormalities).

Some side effects or GI symptoms could occur as an outcome of these dietary interventions; side effects listed in the beverage tolerability questions and reported will not be categorized as AEs but recorded as study outcomes. Side effects, outside of what is expected as a result of study product consumption, reported by participants and judged by the Investigators as medically relevant events and related to study product will be recorded as AEs*.*

Events should be considered AEs if they:

- Result in discontinuation from the study,
- Require treatment or any other therapeutic intervention,
- Require further diagnostic evaluation (excluding a repetition of the same procedure to confirm the abnormality),
- Are associated with clinical signs or symptoms judged by the Investigator to have a significant clinical impact.

Grading and Severity

The Investigator will evaluate all AEs with respect to their severity, and record the outcome and action taken on the AE study documents . AEs will be graded as:

Mild: Awareness of symptoms but easily tolerated
Moderate: Discomfort enough to interfere with but not prevent daily activity
Severe: Unable to perform usual activity

Relationship
The Investigator will also judge the likelihood that the AE was related to the study beverage and document this on the appropriate study documents as:

NOT RELATED	This category applies to those adverse experiences which, after careful consideration, are clearly and incontrovertibly due to extraneous causes (disease, environment, etc.).
UNLIKELY	In general, this category can be considered applicable to those experiences that after careful medical consideration at the time they are evaluated, are judged to be, unlikely related to the study beverage.
POSSIBLY	This category applies to those adverse experiences for which, after careful medical consideration at the time they are evaluated, a connection with the study beverage administration appears possible but cannot be ruled out with certainty.
PROBABLY	This category applies to those adverse experiences that, after careful medical consideration at the time they are evaluated, are felt with a high degree of certainty to be related to the study beverage.
DEFINITELY	This category applies to those adverse experiences which, the Investigator feels are incontrovertibly related to the study beverage.

Appropriate therapeutic action and follow-up measures will be performed by the Investigator in accordance with appropriate medical practice standard of care.

Serious Adverse Event Definition/Qualification
A SAE is defined as an AE that results in any of the following outcomes:

- Death (note that death is the outcome of a SAE and the cause of death should be listed as the AE),
- Life-threatening event,
- In-patient hospitalization or prolongation of existing hospitalization,
- A persistent or significant disability/incapacity,
- Congenital anomaly or birth defect,
- Any other important medical event that may not result in death, be life-threatening, or require hospitalization, may be considered a SAE when, based upon appropriate medical judgment, the event may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed above.

In the event of a SAE, the participant may be dropped from the study if the Investigator deems it necessary.

Serious Adverse Event Reporting Instructions

If in the opinion of the Investigator the event meets the criteria of a SAE the following procedures will be followed:

- The Investigator will notify Institutional Review Board (IRB) of the SAE within the parameters and timeframe specified under the IRB Standard Operating Procedures (SOP). An initial report followed promptly by a complete report will be forwarded to the IRB, when applicable.
- If a participant is hospitalized or hospitalization is prolonged due to the SAE, the hospital discharge summary will be obtained if possible.
- If a death occurs and an autopsy is performed, a copy of the autopsy report will be obtained if possible.
- All efforts must be undertaken to obtain follow-up information promptly.

Recording of Adverse Events

All AEs (AE or SAE) will be recorded on the AE study documents. For participants who have an ongoing AE at their final study visit, follow-up information will be captured in the AE eCRF page which will be completed after 30 days.

Serious Adverse Event Follow-Up

For all ongoing SAEs occurring during the study, the Investigator must submit follow-up reports regarding the participant's subsequent course. All SAEs that are ongoing at the end of the study or upon discontinuation of the participant's participation must be followed until either:

- The event resolves, or
- The event/condition has stabilized (e.g., in the case of persistent impairment), or
- The event returns to baseline, if a baseline value is available, or
- The participant dies, or
- The event can be attributed to other than the study beverage, or to other than the study conduct.

CONDUCT OF THE STUDY

1. **Ethics and Regulatory Considerations**

This study will be conducted according to Good Clinical Practice Guidelines, the Declaration of Helsinki (2004) and United State Code of Federal Regulation Title 21. Signed written informed consent for participation in the study will be obtained from all participants before protocol-specific procedures are carried out. Participants will be

informed of their right to withdraw from the study at any time. Participants will be informed that their participation in the study is completely voluntary, personal information will be both deidentified to preserve anonymity.

2. **Institutional Review Board**

The Investigator will ensure that an appropriately constituted IRB, in compliance with the requirements of 21 CFR 56, reviews and approves the clinical study. Before the study is started, the Investigator will forward copies of the protocol and consent form for this study to the IRB for review and approval. IRB approval must refer to the study by exact protocol title and number, identify the documents reviewed, and state the date of review. The IRB must be informed of all subsequent protocol amendments. No alterations, modifications to IRB-approved documents, including the protocol, protocol summary, consent form, recruitment materials and questionnaires will be allowed. The IRB must also be informed of all SAEs and of unexpected AEs as outlined in the IRB's SOPs or reporting guidelines.

3. **Informed Consent and Protected Health Information**

The study will be explained verbally as well as on the informed consent document. Each participant will be given ample opportunity to inquire about details of the study and to read and understand the consent form before signing it. It will be made clear that participants can withdraw from the study at any time.

Each participant's signed informed consent document must be kept on file by the Investigator. The participant should receive a copy of the informed consent document. A participant may not be admitted to the study unless informed consent of the participant (or his/her legally authorized representative) has been obtained.

4. **Participant Confidentiality**

The Investigator is responsible for ensuring that participants' anonymity will be maintained. For all the data collected over the course of the study for each participant (i.e. records, biological samples and questionnaires) a unique subject identifier (i.e. a code) will be assigned and used instead of the subject's name. The code for each participant which links the subject name with their identifier will only be available to research personnel. Electronic CRFs or other documents will identify participants by initials, number, or code, and not by name. The Investigator will keep a separate log showing codes, names, and addresses. Any records that contain the subject's name and identifier will either be stored in the Kinesiology file storage room in a file cabinet (locked) or protected on a computer via password protection on the individual digital file and password protection on the computer the file(s) are stored on. All other records that contain the subject identifier only will also be kept in either a file cabinet in our locked file storage room or on a password protected computer. Subject names will never be used in

any presentation or publication resulting from this study. The records will be maintained until the data are published and up to a maximum of ten years after the completion of the study. All records or biological data obtained after signing of the informed consent (including the screening visit, even for subjects that are not eligible for participation in the study) are treated with the same confidentiality safety measures as those subjects who qualify. Any information obtained during the prescreening for participants that were not eligible will be deleted

5. **Withdrawal of Participants from the Study**

Participants may be removed from the study for any of the following reasons:

- A participant requests discontinuation;
- The Investigator initiates removal for medical or compliance reasons;
- Occurrence of any AE or condition that could, in the Investigator's opinion, interfere with the evaluation of the effect of the study beverage or put the participant at undue risk.

It is understood by all concerned that an excessive rate of withdrawals can render the study uninterpretable, therefore, unnecessary withdrawal of participants should be avoided. Should a participant decide to withdraw, all efforts will be made to complete and report observations as thoroughly as possible. In the event that a participant is withdrawn from the study, the reason for the withdrawal will be documented in the eCRF.

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