Protein Bioavailability of Wolffia Globosa (Mankai); Acute Test Meal Effects

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Statistical analysis plan and study protocol

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Study Protocol and Statistical analysis plan

Background

Plant protein sources are considered qualitatively inferior to those of animals in terms of their essential amino acid (EAA) composition. Plant sources exhibit less protein digestibility and thus amino acid (AA) bioavailability, some due to their inclusion of trypsin inhibitors or hemagglutinins. In animal models, trypsin inhibitors caused pancreatic hypertrophy and increased synthesis and secretion of proteases (such as trypsin, chymotrypsin and elastase). As a result, the endogenous amino acids are diverted to synthesis of enzymes due to their increased production(1–3). However, unlike plant food sources, red meat products, and particularly processed ones, have been implicated in increased cardio-metabolic risks and diabetes(4–9). The above and other observations have led to an increased movement towards plant-based diets(10), either as a sole nutritional source (e.g. vegetarians) or with occasional inclusion of meat (e.g. "flexitarians"). The question that arises is whether plant sources on their own can provide the requisite EAAs for human nutrition.

An emerging aquatic plant protein introduced to the Western diet is from the duckweed family(11,12), *Wolffia*, a genus within the duckweed family, has been characterized as a rich source of certain EAA compared to seeds(13). Moreover, *Wloffia* has a long history in Southeast Asia as a natural food source or "vegetable meat ball"(12). The morphological structure of *Wolffia globosa* (14,15) is a simple, rootless thalus 0.4-0.9 mm long (the smallest plant on earth), without veins, having a budding pouch at its basal end. Clonal clusters are arranged either solitary or two connected, consisting of a mother plant and a daughter plant. The nutritional properties of Mankai, a cultivated strain of *Wolffia Globosa,* is reflected in high protein content (more than 45%) of the dry matter, the presence of 9 essential and 6 conditional AAs and demonstrable protein digestibility corrected amino acid score [PDCAAS] of 89%.

Main objective

We aim to explore the EAAs bioavailability of Mankai intake in healthy humans, compared to intake of iso-protein containing meals of well-established animal (soft cheese) and plant (peas) protein sources.

Experimental design

Three days prior to the test meal, the participants will be instructed to maintain a stable diet that consist of detailed recipes tailored to provide 2,000 kcal/day, comprised of 55% carbohydrates, 15% protein, and 30% fat from total energy.

Baseline blood samples will be taken after a 12h overnight fast by four paramedics and, following meal administration, after 30, 90 and 180 minutes. To keep exact timeintervals, each randomized intervention group will be further divided into three time groups, with baseline sampling at 8 am, 8.15 am, 8.30 am, and the exact time sequence will be maintained throughout the entire study. This study is a single-phasetrial.

Each test meal will provide 30 gr of protein from the respective source: 1. Cheese protein (333gr of soft white cheese, 9% fat) 2. Green peas (cooked, cutlet) 3. *Wolffiaglobosa* (Mankai, cooked, cutlet). A chef prepared the cutlets that included 30gr protein from either peas (600gr) or Mankai (410gr) with an additional minimal list ofingredients. The final recipe included the following additional ingredients for both cutlets: onions (280 gr), mushrooms (200 gr), canola oil (21gr for peas, 58.2gr for Mankai), protein-free stabilizer (16.2gr for peas, 13.4gr for Mankai), salt and pepper.The cheese meal will include one slice of bread. In addition to the 30gr equivalent protein content of each protein specific source, the estimated amount of protein provided in each dish wil be as follows: cheese (39.7gr), peas (39.3gr) and Mankai (39.3gr). While other nutritional values/components varied across groups, the 3 test meals groups have

a complete profile of all the 9 EAAs.

All the test meals will be served with a 250ml bottle of mineral water. To maintain an exact time frame, the participants will be required to complete their meal within 15 minutes at their allocated food tables. Blood samples will be taken at baseline, and at 30, 90, and 180 minutes post administration of the test meals for the EAAs measurements. Other parameters will be measured only in 3 hours interval.

Statistical analysis plan

The primary outcome is the change of the EAAs concentration in the blood. The secondary outcomes are selected blood biomarkers, as B12 vitamin, which is primarily derived by animal protein sources. Computer-generated random numbers will be used to assign the subjects to the three different meals randomly. All the statistical analyses will be based on non-parametric tests. Differences in blood measures at various time points, as compared to baseline, will be analyzed by the Friedman or Wilcoxon test, depending on the number of time points. To examine the differences between the three meal groups, we will use the Kruskal-Wallis test. In case of significant differences between groups, we will use Mann-Whitney test as a post-hoc test. In a sensitivity analysis, the data will be further tested by using general estimating equation (GEE) model with a random effect for subjects and fixed effect ofmeal groups. Statistical analyses will be performed with IBM SPSS (version 24). All *P*-values are two-sided, and $P<0.05$ is considered statistically significant.

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