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A Prospective, Single Blind RCT to Assess the Acute Effect of Protein Rich Oral Nutritional Supplements on Serum Levels of Amino Acids in Healthy Indian Adults

Deepti Khanna[1*], Hema S. Gopalan[1*], Manoj Karwa[2], Sanjay Kumar Mishra[2], Jaladhi Bhatt[1], Minakshi S. Singh[1], Surya Teja Reddy[2]

[1]R&D Department, Hindustan Unilever Limited, Gurugram, India
[2]Auriga Research Private Limited, Gurugram, India
Email: deepti.khanna2@unilever.com

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Abstract

Indian diets are protein deficient. Our population is physiologically predisposed to lower muscle mass. There are many protein-rich oral nutrition supplements (P-ONSs) available to support this condition. A single-blind, randomized study was designed to evaluate the acute effect of two supplements: P-ONS1 and P-ONS2 on serum amino acid (AA) levels in healthy Indian adults. Twenty-four participants were randomized to receive either P-ONS1, P-ONS2 or energy matched placebo (control), all in milk. In this three-period crossover study, after a 7-day washout, the participants crossed over to the next supplementation arm. Blood samples were collected at 0-, 15-, 30-, 60- and 90-minutes post P-ONS/placebo consumption. Serum AA levels were estimated using LC-MS and outcome parameters of Cmax (maximum serum AA concentration) and Area Under curve (AUC) were analyzed. N = 23 participants completed the study. Cmax was observed to be significantly higher for total AAs (TAAs), essential AAs (EAAs) and branched chain AAs (BCAAs) for P-ONS1 ($p = 0.0009$, $p = 0.0245$ and $p = 0.0009$ respectively) and P-ONS2 ($p = 0.0086$, $p = 0.0006$ and $p = 0.0009$ respectively) versus placebo. Similarly, AUC for both P-ONS1 and P-ONS2 was significantly higher for TAAs, EAAs, BCAAs ($p = 0.0305$, $p = 0.0133$, $p = 0.02$ and $p = 0.0163$, $p = 0.0017$, $p = 0.0007$ respectively) versus placebo. This suggests significantly better absorption and bioavailability of amino acids in the serum post P-ONS1 and P-ONS2 consumption. No adverse events were reported. It may be concluded that such P-ONSs have a beneficial acute effect on serum AA levels. Long term consump-

*These authors contributed equally to this work.

tion can improve quality and quantity of protein in Indian diets to support a better muscle health.

Keywords

Oral Nutrition Supplements (ONS), Amino Acids, Branched Chain Amino Acids, Area Under Curve (AUC), Absorption Efficacy (Cmax)

1. Introduction

Proteins are comprised of amino acids which serve as fundamental building blocks. More than 300 amino acids have been described, but only 20 amino acids take part in protein synthesis. Amino acids which are not synthesized in the body and need to be taken through the diet are essential amino acids (EAAs) and those synthesized in the body and not necessary from the diet are non-EAAs. Of the EAAs, three AAs are termed as branched-chain amino acids (BCAAs‑leucine, isoleucine, valine) [1].

Asian Indian diets are characterized by a high consumption of cereals with lower intakes of protein, fruit, and vegetable [2]. The dependence on cereal-based diets to fulfil the body's protein needs is considered inadequate, as cereal proteins are of poor quality and digestibility. A report on food consumption patterns in India, stated that cereals were the primary source of protein intake, providing 28.2 g and 41 g of protein/day in urban and rural areas, respectively; that is, cereals accounted for 39% of the total daily protein intake for urban and 61% in rural areas. Pulses and flesh foods collectively contributed 23.4% and 10.8% of the total protein intake in urban and rural India, respectively [3]. In India as vegetarianism is quite prevalent [4] [5], and to meet the daily protein requirements, milk and dairy products are commonly consumed as a source of "complete protein". The ICMR-NIN report, "What India Eats," reveals that only 7.6% of protein intake in urban and 5.5% in rural areas comes from milk and milk products. Additionally, just 5% of rural and 18% of urban populations consume recommended levels of good quality protein [6]. This clearly indicates that Indians are not consuming enough high-quality protein.

Consequently, to meet the daily protein requirements of the body, it is possible that excessive amounts of carbohydrates are consumed in the form of wheat flour/rice, that would eventually be converted into fat in the body, thereby contributing to development of obesity, dyslipidemia, and a cause of metabolic diseases [7]. The Pune Maternal Nutrition study by Yagnik *et al.* showed that adult Indian phenotype of excess total and central body fat in a relatively "thin" individual may originate in utero and predispose Indian men and women to insulin resistance syndrome [8]. Indians exhibit the "thin fat" phenotype [9] [10] that leans towards fat accumulation implying increased visceral adipose tissue and decreased lean mass. Indians are reported to have lower skeletal muscle mass ("sarcopenia") than their western counterparts [11]. Young adults also possess significantly lower muscle strength compared to the western population, despite having a comparable bone cell mass (BCM) [12]. A study by Marwaha *et al.* in women, both pre and post-menopausal, found that mean appendicular lean mass increased from the second decade to reach maximum in the fourth decade in Indian women after which it declined. Post-menopausal females with low muscle mass were significantly older, leaner, and had lower bone mineral density (BMD) [13]. Further, it is reported that muscle mass decreases by 3% - 8% per decade after 30 years, accelerating after 60 years, and can be a cause for disability in older adults [14]. Additionally, studies have demonstrated that a vegetarian diet is associated with a lower muscle mass index than an omnivorous diet with same protein intake in older women [15]. A hospital-based assessment of sarcopenia showed that Indian men were eight times more likely to be sarcopenic than women [16].

While organs such as the heart, liver, pancreas, and bones receive a lot of attention, skeletal muscle, comprising 40% of body weight, is often overlooked. Any decline in motor abilities or aging can lead to muscle atrophy, resulting in low energy expenditure (causing obesity), increased blood glucose levels, and overall lower quality of life [17]. Muscle serves as a reservoir for amino acids used by the body for various functions and deserves more consideration. Therefore, it is amply evident that the natural aging process, poor dietary protein sources, and inadequate physical activity [18] often lowers the quality of life when Indian adults enter their senior years with inadequate muscle mass [7]. Consequently, the risk of developing chronic diseases and susceptibility to falls/fractures increases. All the above factors emphasize the crucial need for incorporating diverse and adequate dietary protein sources to meet the nutritional needs of the population.

The nutritionally EAA requirements for Indian adults have been provided by the Indian Council of Medical Research [19]. There are protein-rich oral nutrition supplements available which are scientifically designed to bridge the gap in the nutritional requirements of proteins and EAAs. There are several studies that have looked at long term effects of ONSs on muscle structure, function, and role in energy and protein balance, and have reported benefits around muscle mass and strength. Based on sarcopenic severity, changes observed were increases in lean muscle mass, improved muscle quality, grip strength, gait speed, lower and upper body strength [20]-[27]. As for the short term or acute effects of consuming protein rich ONSs on the serum amino acid pool, there is limited data [28] for the Indian adults/elderly.

This single-blind, randomized, crossover study intended to observe the acute effects of two protein rich ONSs (P-ONS1 and P-ONS2) on the serum amino acids, their absorption and bioavailability in healthy Indian adults when compared against an energy matched placebo (milk).

2. Material and Methods

This single-blind randomized, crossover study was conducted on 24 healthy adults to assess the acute effects of P-ONSs on serum amino acid levels. This study was conducted between Jan-Apr 2024. It was performed in accordance with the protocol (NUN-HFD-006/23 Version 1.0), principles and requirements of the Declaration of Helsinki and was consistent with the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guidance on Good Clinical Practice (2019) [29] and Indian Council of Medical Research ethical guidelines. The study was reviewed and approved by the Independent Ethics Committee "Good Society Ethical Research" on 11th December 2023. All participants provided written informed consent. The study was registered at Clinical Trials Registry India with registration number CTRI/2023/12/060650 (Available at CTRI https://ctri.nic.in/Clinicaltrials/pmaindet2.php?EncHid=OTY2MDI=&Enc=&userName=).

2.1. Study Flow and Participants

The primary objective of this study was to investigate the acute effects of two protein rich ONSs on serum AA in human participants. A total of N = 24 participants were randomized into three groups labelled as A, B, and C with n = 8 participants in each arm (Refer to Figure 1).

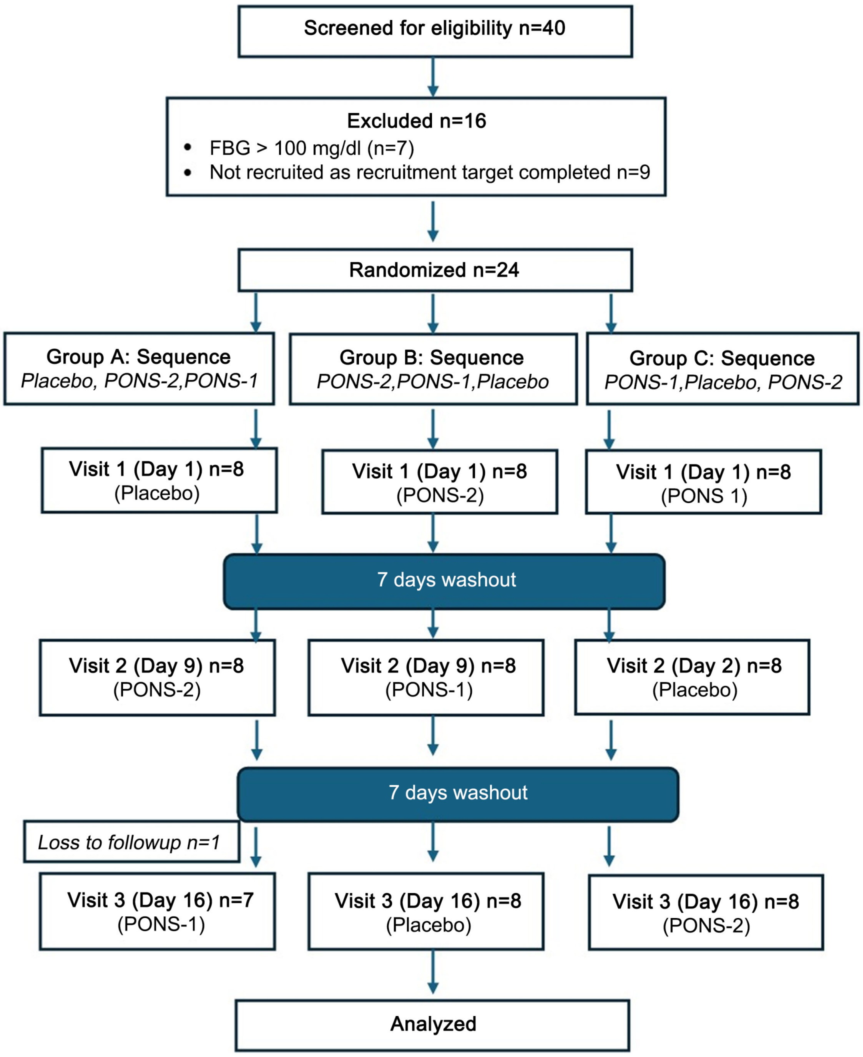

Figure 1. CONSORT diagram.

On visit 1, all participants were required to report to the study facility after an overnight fast (8 - 12 hours). Vital signs of body temperature, pulse rate, and blood pressure were recorded. An intravenous cannula was inserted, and pre-dose samples were collected in the fasting state. 5 ml blood samples were collected in pre-labelled serum separator tubes (SST) vacutainers. The vacutainers were then centrifuged at 4000 RPM for 10 minutes at 5˚C ± 3˚C to separate the serum. After centrifugation, the serum samples were transferred into pre-labelled polypropylene tubes, in duplicates (analytical and replicate), as soon as possible. These tubes were then stored in a deep freezer maintained at −20˚C or below immediately until further analysis. Post baseline data, the three drinks were prepared by adding 30 g of powder from sachets to 200 ml of lukewarm milk (low-fat) in a calibrated shaker and stirred until well blended. The drink was consumed at room temperature (within 5 minutes of preparation), in a sitting position and under the supervision of trained study personnel based on randomization schedules. Blood samples were collected at 15, 30, 60, and 90 minutes after the consumption of the P-ONS/placebo. Previous research has clearly reported maximum amino acid concentration in the blood at 60 minutes post-ingestion of a protein supplement after which it plateaued till 120 minutes [28]. As the objective of this study was to mainly observe the acute effect of P-ONSs, blood samples were collected till 90 minutes. Each separated serum sample were transferred in separate, stoppered and suitably labelled storage vials mentioning protocol number, participant number, period, time point etc. No information regarding the identity of product administered was mentioned on the labels.

Hence, it was ensured that the analyst was blinded to the treatment administered. Samples were transported to the laboratory and stored at −20˚C or below until further analysis. After a 7-day washout, the participants returned to the study facility for the next crossover treatment. The same process was followed for visits 2 and 3 as well.

Each participant received all three drinks-P-ONS1, P-ONS2 and energy matched placebo (control) thereby serving as his/her own control. All the three powders were packed in identical, blank and unbranded sachets which were only coded. The drinks were prepared before the participants arrived at the center. The participants remained blind in the study as they consumed the drink in opaque shakers and were not aware of the drink/its appearance. The study included both men and women (non-pregnant/non-lactating) aged between 25 to 60 years with a Body Mass Index (BMI) between 20 - 30 kg/m^2 (where weight was ≥50 kg). Enrolled participants were required to have normoglycemia (ADA guidelines), with fasting serum glucose (FPG) < 100 mg/dl. Participants were excluded if they had food allergies, drug abuse, alcoholism, smoking, diabetes, certain medication used, recent illness or hospitalization, gastrointestinal, liver, or kidney disease, peptic ulcers, or conditions affecting drug metabolism. Additionally, those with resting hypotension (BP < 90/60), hypertension (BP > 139/89), pulse rates below 50/min or above 99/min, or those following protein-rich diets or bodybuilding programs were excluded. Potential participants who were related or lived together were not recruited together; one was excluded. Employees of the sponsor or the study site were also excluded.

2.2. Details of P-ONS 1, 2 and Placebo

P-ONS1 and P-ONS2 are protein rich ONSs designed to suit the nutritional requirement of Indian adults, with P-ONS1 being focused on nutritional requirements of elderly (60+ years). These ONSs are designed to supplement the traditional Indian diets by providing a balanced mix of macro and micronutrients. Both P-ONS1 and P-ONS2 are manufactured and marketed by Hindustan Unilever Limited, India (P-ONS1-Horlicks Strength Plus; P-ONS2-Horlicks Protein Plus). P-ONS1 contains a double blend of whey and casein protein with added leucine for additional muscle health benefits. P-ONS2 features a triple blend of whey, casein, and soy protein. P-ONS1 and P-ONS2 provided different types and amounts of protein (8.1 g and 10.2 g respectively) with an additional 6.6 g of protein provided by milk to both the test groups. The control was an energy matched placebo (maltodextrin powder). All three products were dispensed as 30 g sachets to be consumed in 200 ml lukewarm milk (low fat). Milk is a staple in vegetarian Indian diets and a natural source of essential amino acids. It is considered a gold standard and an ideal quality protein source. The two P-ONSs delivered different amounts of protein. The objective of the study was to observe the acute effect these P-ONSs had on the absorption/bioavailability of amino acids as they are typically consumed (*i.e.* in milk), hence matched levels of milk was used as the medium of consumption across the three groups. Hence, an energy matched placebo was considered the best fit for this study.

The nutrient composition details of P-ONS1 and 2 along with placebo are provided in **Table 1**. As these ONSs are rich in protein, they are also expected to be rich in amino acids. To illustrate this, analytical analysis of the AA content of the two P-ONSs was undertaken via *in vitro* testing. Post analysis, the levels of AAs delivered by 30 g of P-ONS1 and 2 in 200 ml milk were compared with daily AA intakes recommended by ICMR 2020. This comparison indicated that both P-ONS1 and 2 provide 50% - 100% of the daily EAR of most of the EAAs. The analytical process as well as % EAR table is detailed in Supplementary materials (Supplementary **Box S1** and **Table S1** respectively).

Table 1. Comparison of nutrition composition of P-ONS1, P-ONS2 and energy matched placebo.

Nutrient	Units	P-ONS1 + milk*	P-ONS2 + milk*	Energy matched placebo + milk*
Energy	Kcal	211.0	206.2	210.4
Carbohydrates	G	25.0	25.6	38.5
Protein	G	14.7	16.8	6.6
Fat	G	6.0	4.2	3.0

*30 g powder added to 200 ml low fat milk.

2.3. Targeted Amino Acid Analysis

Amino acid analysis was performed by using AccQ-Tag Ultra kit (Waters, Massachusetts, USA) and UPLC-MS system in the serum/serum samples. Wet ice baths were used for storage of samples from blood sample collection to centrifugation and sample freezing. The temperature of wet ice bath was maintained and monitored below 10˚C. Samples were handled in normal light conditions and stored at or below to −20˚C. AAs selected for analysis were—Alanine, Arginine, Aspartic acid, Cysteine, Glutamic acid, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tyrosine and Valine. Since obtaining AA free blank biological matrix was not possible, all standard samples were prepared in aqueous/water/solutions suitably stripped of serum/serum matrix and processed samples were analyzed along with amino acid level determination. The sample processing procedure began with the addition of 20 µL of water to a 1 mL low bind Eppendorf vial, followed by the introduction of 20 µL of serum/serum, with subsequent vortexing for 15 seconds. To this mixture, 80 µL of chilled 0.1% formic acid in isopropanol was added, and the sample was vortexed for an additional 30 seconds. The resulting solution was then stored at −20˚C for a period of 20 minutes before undergoing centrifugation at 12,000 rpm for 10 minutes at 4˚C. The supernatants, which contained the target analytes, were carefully separated and reserved for derivatization. 70 µL of Borate buffer, available with the kit, was placed in a total recovery vial. To this, 20 µL of the supernatant (the extracted serum/serum supernatant) was added, and the vial was vortexed for 15 seconds to ensure thorough mixing. Subsequently, 20 µL of Accq-Flour ultra-reagent, which acts as the derivatizing agent, was introduced into the mixture. The solution was once again vortexed, this time for 30 seconds. Following this, the samples were placed in a water bath preset to 55˚C for an incubation period of 10 minutes. After incubation, the samples were retrieved, vortexed again, and allowed to equilibrate at room temperature for an additional 2 minutes. These samples were then loaded into the autosampler and subsequently injected into the UPLC-MS/MS system for comprehensive amino acid analysis. The mobile phase operates with a gradient distribution between Mobile Phase-A (Diluted Eluent-A) and Mobile Phase-B (Eluent-B as is) at a flow rate of 0.50 mL/min. The Accq Tag Ultra column with dimensions of 100× 2.1 mm, with particle size of 1.7 µL was used. For sample injection, 2.5 µL is employed (from a 10 µL sample loop), while the autosampler and column oven are maintained at temperatures of 10˚C and 55˚C, respectively. The total run time for this analysis was 10 minutes, and it was conducted on an Acquity UPLC system coupled with an MS/MS detector, either the Acquity TQD or Quattro Micro. All samples collected were analyzed and reported. The concentration of each amino acid in the samples and TAAs were reported. All quality measures were taken for the batch, and results in acceptance were applicable as per in-house SOPs. All samples from one participant were analyzed in one batch run wherever applicable. The analyst did not have access to randomization.

Two pharmacokinetic (PK) parameters were estimated:

1) Cmax: Maximum measured serum concentration over the time span specified. This parameter is an indicator of AA absorption efficacy.

2) AUC_{0-t}: Area under the serum concentration versus time curve, from time 0 to the last measurable concentration, where t = time of last identifiable concentration, calculated using linear trapezoidal method. This parameter is an indicator of AA bioavailability.

2.4. Statistical Analysis

The primary objective was to assess the efficacy of the two P-ONSs versus placebo. Considering an intra-participant C.V. of 16%, 5% significance level, and 90% power, the sample size required for the study was 17, with consideration for a 20% - 25% dropout rate. Consequently, the study was planned to recruit 24 participants. The sample size was calculated based on a study by Burke *et al.* [30]. Data collection adhered to Good Clinical Practice (GCP) standards. Demographic and baseline characteristics, including age, sex, height, weight, were summarized using appropriate statistical measures such as mean, standard deviation, median, and range for continuous variables, and proportions for categorical data. Statistical examinations utilized both descriptive and independent T-tests for comparing groups. Significance was determined at $p < 0.05$ and elevated significance at $p < 0.01$, with explicit delineation of hypotheses. Plots were generated as necessary, such as plotting individual AAs, TAAs, EAAs and BCAAs levels on the Y-axis against time-points on the X-axis. All the analyses were performed using Phoenix WinNonLin, Version 8.4 Using the serum concentrations, additional analysis on the rate of absorption by calculation of slope was done to interpret the vertical change (rise) in concentration of the AA in the blood between two time points. The slope (m) of a line is the measure of its steepness and is calculated as the ratio of the vertical change (rise) to the horizontal change (time-run) between two points on the line. The formula for the slope m is given by:

$$m = y_2 - y_1/x_2 - x_1$$

where (x_1, y_1) and (x_2, y_2) are the coordinates of two distinct points on the line. y_2 and y_1 correspond to the concentration of serum amino acids levels at 15 & 30 mins respectively. x corresponds to the time with x_2 as 15 or 30 mins and x_1 as 0 mins. Slope (m) calculated as the ratio of difference between amino acid concentrations at t_0 and t_{15} or t_{30} represented as m_{15} and m_{30} respectively.

3. Results

A total of 24 participants were enrolled and 23 successfully completed the study. The mean age and BMI of the participants were 35.6 ± 6.50 years and 24.9 ± 2.35 kg/m^2, respectively. The three groups were comparable at the start of the study (Table 2).

Cmax or absorption efficacy

The maximum serum concentration of AAs over the 90 minutes period referred to as the Cmax or absorption efficacy was observed for P-ONS1, P-ONS2 and placebo (all consumed in milk).

Table 2. Demographic characteristics of participants in each group.

Characteristic	P-ONS1 (n = 8)		P-ONS2 (n = 8)		Placebo (n = 8)		Overall P Value
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	
Age (Years)	36.50	5.83	35.88	7.68	34.38	6.57	0.8120
Height (cm)	166.25	6.25	167.13	6.56	170.13	5.00	0.4117
Weight (Kg)	66.63	6.46	71.38	8.57	71.75	5.09	0.2251
BMI (kg/m^2)	24.40	2.99	25.55	2.44	24.81	1.55	0.6311
Gender	**n**	**%**	**n**	**%**	**n**	**%**	**NA**
Male	7.00	87.50	7.00	87.50	8.00	100.00	NA
Female	1.00	12.50	1.00	12.50	0.00	0.00	NA

Note: Overall P value calculated using ANOVA: Single Factor.

Total amino acids: Amongst the non-EAAs, tyrosine and proline showed significantly higher (p = 0.0130 and p = 0.0482) Cmax respectively for P-ONS1 versus placebo. P-ONS2 also reported a significantly higher Cmax for tyrosine versus placebo (p = 0.0128). Glycine and serine also reported trends of higher Cmax for P-ONS1 versus placebo but missed significance (p = 0.0588 and p = 0.0822 respectively). Figure 2(a) depicts the sum of peak concentration of TAAs for each of the 3 groups P-ONS1, P-ONS2 and placebo over time points 0, 15, 30, 60 and 90 mins. It was observed that both P-ONS1 and 2 had significantly higher sum of peak concentration (p = 0.0009, p = 0.0086) versus placebo (Table 3 and Figure 2(a)).

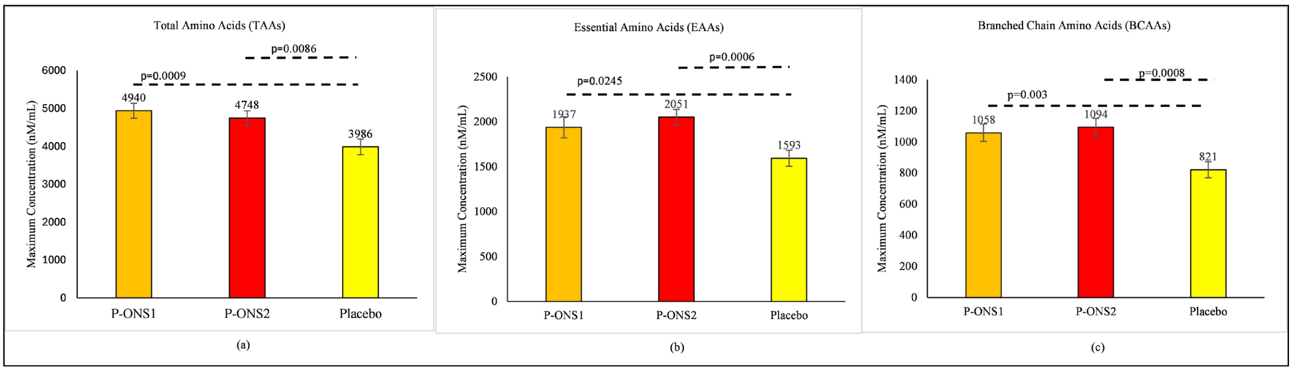

Figure 2. Mean sum of peak concentrations (Cmax) (Mean ± SE) of plasma levels of TAA, EAA and BCAA in P-ONS1, P-ONS2 and placebo.

The percentage increase of TAAs from baseline to Cmax was calculated and indicated a steeper rise in the concentration of TAAs for P-ONS1 and 2 being 29.0% and 36.1% respectively versus a smaller 7.7% increase in serum AA concentration for placebo (Figure 3). For TAAs, the rate of absorption was observed to be higher for P-ONS1 (≈3 times) versus placebo. Similarly, for P-ONS2, it was 2 times versus placebo at 15 minutes and increased 5 times versus placebo at 30 minutes (Table 4).

Table 3. Comparison of peak concentration (Cmax, Mean ± SE) of TAAs, EAAs and BCAAs with relative absorption for P-ONS1, P-ONS2 versus placebo.

Amino acids	Cmax Mean (nM/mL)			Significance P-ONS1 vs. placebo	Significance P-ONS2 vs. placebo	Relative Absorption	
	P-ONS 1	P-ONS 2	Placebo			P-ONS1/ placebo (%)	P-ONS2/ placebo (%)
TAAs	4939.7 ± 197.9	4747.5 ± 187.0	3985.6 ± 205.0	0.0009**	0.0086**	123.9 (24)	119.12 (19)
EAAs	1936.9 ± 117.0	2050.5 ± 85.5	1593.0 ± 90.3	0.0030**	0.0008**	121.6 (22)	128.7 (29)
BCAAs	1057.5 ± 56.4	1094.1 ± 56.7	820.5 ± 52.0	0.0009**	0.0009**	128.9 (29)	133.3 (33)

**Highly significant at $p < 0.001$; Abbreviations: Cmax—Maximum Serum Concentration; TAA—Total Amino Acids; EAA—Essential Amino Acids; BCAA—Branched Chain Amino Acids; P-ONS—Protein rich Oral Nutrition Supplement.

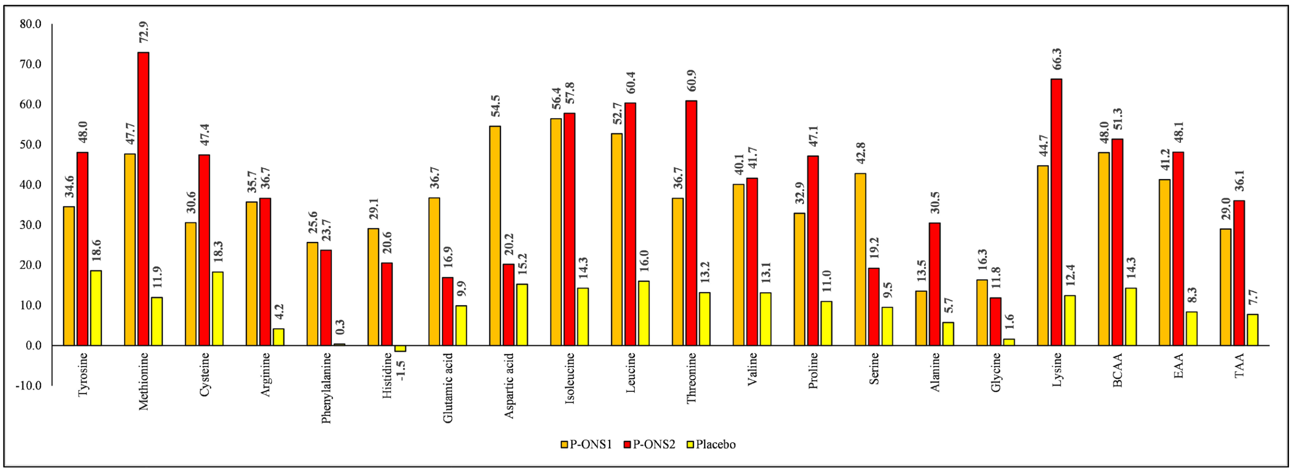

Figure 3. Percentage increase of plasma amino acids from baseline to Cmax for P-ONS1, P-ONS2 and placebo.

Table 4. Rate of absorption or gradient of TAAs, EAAs AND BCAAs in P-ONS1 and P-ONS2 and comparison with placebo.

Plasma amino acid concentration	Time (in minutes)					Slope		Ratio of m_{15} and m_{30}: ONS/control	
	0	15	30	60	90	m_{15}	m_{30}	15 mins	30 mins
					TAAs (nM/mL)				
P-ONS1	3171.16	4018.18	4090.85	3927.14	3910.75	56.47	30.66	3	5
P-ONS2	3152.19	3772.46	4106.00	4288.58	3975.53	41.35	31.79	2	5
Placebo	3349.63	3609.11	3538.82	3320.45	3376.59	17.30	6.31	-	-
					EAAs (nM/mL)				
P-ONS1	1162.41	1528.68	1641.89	1535.46	1588.55	24.4	16.0	3.3	5
P-ONS2	1216.88	1574.2	1773.77	1802.45	1709.51	23.8	18.6	3.2	5
Placebo	1317.97	1428.02	1420.52	1288.22	1301.45	7.3	3.4	-	-
					BCAAs (nM/mL)				
P-ONS1	604.91	832.34	895.12	824.81	871.02	15.2	9.7	3.6	3
P-ONS2	629.50	810.16	944.32	952.73	879.20	12.0	10.5	2.9	3
Placebo	647.19	710.15	739.57	644.99	654.83	4.2	3.1	-	-

Notes: y: concentration of plasma amino acids levels at 15 & 30 mins; x: Time with x_2 as 15 or 30 mins and x_1 as 0 mins; m: Slope calculated as ratio of difference between amino acid concentrations at t_0 and t_{15} or t_{30} represented as m_{15} and m_{30} respectively; Abbreviations: TAA—Total Amino Acids; EAA—Essential Amino Acids; BCAA—Branched Chain Amino Acids; P-ONS—Protein rich Oral Nutrition Supplement.

These results suggest that both P-ONS1 and 2 are considerably more effective than milk in terms of the extent and rate of absorption for serum AA levels, suggesting better absorption efficacy. The relative absorption of TAAs was 24% and 19% for P-ONS1 & 2 respectively when compared with milk (Table 3).

EAAs & BCAAs: Seven of the eight EAAs analyzed reported significant differences for Cmax (mean ± SE) for both P-ONS1 and P-ONS2 versus placebo: isoleucine ($p = 0.0007$; $p = 0.0007$), leucine ($p = 0.0007$; $p = 0.0007$), lysine ($p = 0.0226$; $p = 0.0007$), methionine ($p = 0.0047$; $p = 0.0003$), phenylalanine ($p = 0.0371$; $p = 0.0300$), threonine ($p = 0.0007$; $p = 0.0007$), and valine ($p = 0.0307$; $p = 0.0141$) (Supplementary Table S2). While in P-ONS1 histidine versus milk missed significance ($p = 0.0795$), it reported trends of higher Cmax and better absorption for amino acid. Figure 2(b) & Figure 2(c) depict the sum of peak concentration of Cmax for EAAs and BCAAs for P-ONS1, P-ONS2 and placebo over time. The Cmax reported for EAA for both P-ONS1 and P-ONS2 were significantly higher ($p = 0.0245$, $p = 0.0006$) respectively versus placebo. Similar results were reported for the sum of peak concentration of BCAAs for P-ONS1 and 2 ($p = 0.0009$, $p = 0.0009$) respectively versus placebo. The percentage increase of EAAs from baseline to Cmax was also calculated for all three study groups and indicated a steeper rise in the concentration of EAAs for P-ONS1 and 2 being 41.2% and 48.1% respectively versus a smaller 8.3% increase in serum EAA concentration for placebo. Similar findings were reported for BCAAs to be 48% and 51.3% higher than placebo at 14.3% (Figure 3). For other AAs, methionine, threonine, phenylalanine, histidine and lysine increments ranged from 25.6% to 47.7% for P-ONS1 and 20.6% to 70.9% for P-ONS2. The highest percentage increase for any AA from baseline was reported by methionine in P-ONS2 (70.9%) versus 11.9% in placebo. This difference was notably observed to be 6 times in P-ONS2. EAAs in both P-ONS1 and P-ONS2 had a rate of absorption of 3 and 5 times that of placebo at 15 and 30 minutes (Table 5). These results indicate a better nutritional efficacy of both P-ONS1 & 2 when compared to a standard food item like milk. For EAAs and BCAAs the relative absorption was 22% and 29% respectively for PONS1 and 29% and 33% respectively for PONS2 (Table 2).

Table 5. Comparison of AUC0-t (Mean ± SE) of TAAs, EAAs and BCAAs with relative bioavailability for P-ONS1, P-ONS2 versus placebo.

Amino acids	Mean AUC (nM/mL.hr)			Significance P-ONS1 vs. placebo	Significance P-ONS2 vs. placebo	Relative bioavailability	
	P-ONS1	P-ONS2	Placebo			P-ONS1/ placebo (%)	P-ONS2/ placebo (%)
TAAs	5876.26 ± 200.4	6015.06 ± 236.6	5152.41 ± 252.3	0.0133*	0.0017**	114.1 (14)	116.7 (17)
EAAs	2408.40 ± 92.8	2539.43 ± 102.5	2023.92 ± 115.7	0.0305*	0.0163*	119.0 (19)	125.5 (26)
BCAAs	1249.53 ± 63.4	1331.51 ± 63.4	1021.98 ± 67.6	0.0200*	0.0007**	122.3 (22)	130.3 (30)

*Significant at $p < 0.05$; **highly significant at $p < 0.001$; Abbreviations: AUC—Area Under Curve; TAA—Total Amino Acids; EAA—Essential Amino Acids; BCAA—Branched Chain Amino Acids; P-ONS—Protein rich Oral Nutrition Supplement.

AUC *i.e.* Area Under Curve (Serum Concentration)

The AUC *i.e.* area under the curve versus time, which is also an indicator for bioavailability, was calculated for all individual amino acids for the 90 minutes period. The bioavailability was observed for P-ONS1, P-ONS2, and energy matched placebo (all consumed in milk).

Total amino acids: The mean AUC for TAAs for both P-ONS1 and 2 were significantly higher (p = 0.0133, p = 0.0017) versus placebo (Table 5). Tyrosine showed significant differences (p = 0.0413; p = 0.0099) for AUC (mean ± SE) vs. placebo for both P-ONS1 and 2 respectively (Table S3). Similarly, proline also reported higher trends for AUC for P-ONS2 versus placebo but missed significance (p = 0.0787) (Supplementary Table S3). The mean sum of AUC of serum TAAs in P-ONS1, P-ONS2 and placebo is shown in Figure 4(a). The sum of AUC was significantly higher for P-ONS1 and 2 versus placebo for all TAAs (p = 0.0305, p = 0.0163). TAAs were observed to be 19% more available for uptake in circulation than milk in the case of P-ONS1 and 26% more available for uptake in circulation than milk in the case of P-ONS2 (Table 5).

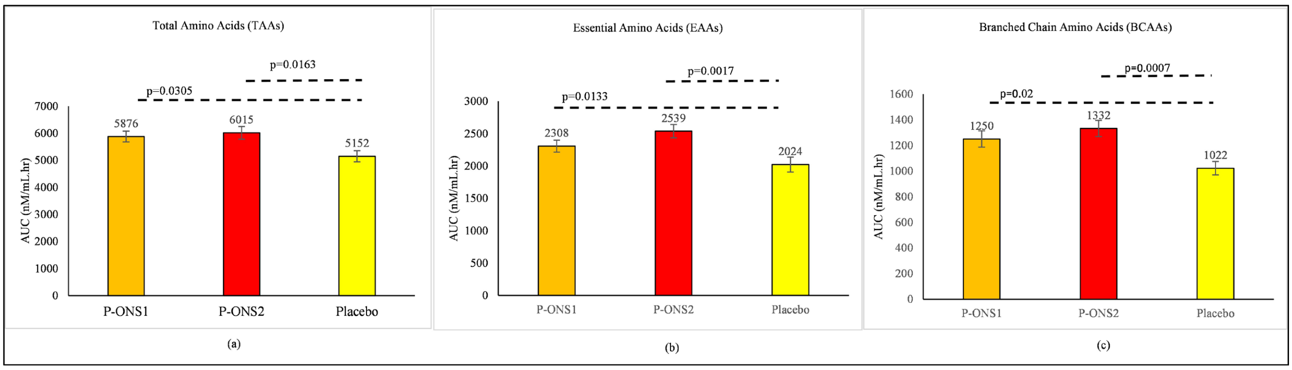

Figure 4. Mean sum of AUC (mean ± SE) of plasma levels of TAA, EAA and BCAA in P-ONS1, P-ONS2 and placebo.

EAAs and BCAAs: Four of the eight individual EAAs analyzed reported significant differences for AUC (mean ± SE) for PONS1 versus placebo namely methionine (p = 0.0202), threonine (p = 0.0007), isoleucine (p = 0.0053) and leucine (p = 0.0053), with isoleucine and leucine being BCAAs. Six out of eight EAAs reported significant differences in AUC of P-ONS2 versus placebo namely methionine (p = 0.0003); valine (p = 0.0346), threonine (p = 0.0007), isoleucine (p = 0.0007); leucine (p = 0.0007); lysine (p = 0.0057). Phenylalanine reported higher AUC versus placebo but missed significance (p = 0.0780) in P-ONS2. The significantly different levels of lysine in P-ONS2 could be attributed to soy protein in the protein blend. The mean sum of AUC of serum EAAs and BCAA in P-ONS1, P-ONS2 and placebo is shown in Figure 4(b) and Figure 4(c) respectively. The sum of AUC was significantly higher for P-ONS 1 and 2 versus placebo for EAAs (p = 0.0133, p = 0.0017) and BCAAs (p = 0.0200, p = 0.0007) respectively (Table 5 and Figure 4(b) and Figure 4(c)). Overall BCAAs and EAAs were observed to be 22% and 19% respectively more available for uptake in the circulation than milk in the case of P-ONS1 and 30% and 26% more available for uptake in the circulation than milk in the case of P-ONS2 (Table 5).

4. Discussion

This study was designed to assess the short-term effects of two protein rich ONSs in the Indian population on AA pool, absorption and bioavailability when compared against an energy matched placebo. All the outcome parameters like Cmax, AUC, percent change in AAs from baseline and rate of absorption for TAAs, EAAs and BCAAs have reported significantly higher levels for P-ONS1 and P-ONS2 within the 90-minute window of observation. This indicates a higher absorption and improved bioavailability of AAs post P-ONS1 and 2 consumptions versus milk. The improved pool of AAs could support muscle growth and repair. P-ONS1, with whey protein and added leucine, offers a targeted approach to stimulating muscle protein synthesis (MPS) in senior populations to assist in muscle health and function; P-ONS2 provides higher AAs vs milk, suggesting it's potential as a protein rich nutritional supplement for healthy young adults.

During the formulation of high protein ONSs, the protein source and its quality are vital considerations. High-quality proteins like whey, casein, and soy are preferred for their AA profiles and digestibility. To maintain a high protein source and quality, P-ONS1 is designed with a double blend of whey and casein protein with added leucine. Similarly, P-ONS2 features a triple blend of whey, casein, and soy protein.

For individual AAs, TAAs, EAAs, or BCAAs, the highest mean concentration was observed at 30 minutes post-consumption, indicating rapid and efficient uptake into the serum pool for tissue absorption. For TAAs, the rate of absorption was observed to be higher for P-ONS1 (≈3 times) and for P-ONS2; it was 2 times versus placebo at 15 minutes. The increase in concentration of AAs post consumption indicated a steep slope for P-ONS1 followed by P-ONS2 and finally for the placebo arm. Whey, known as a "fast protein", has the highest absorption rate, followed by casein and then soy protein. The steep increases in gradient observed in P-ONS1 can be attributed to the whey protein in the powder, resulting in a higher Cmax in a shorter time span (15 minutes). A study by Dangin suggested that "fast" protein (whey) may be more beneficial than a "slow" one in elderly participants, to limit body protein loss [31]. It appears the higher percentage of slower digesting proteins casein and soy ~55% of total protein in P-ONS2 are lowering the overall absorption rate compared to P-ONS1, which has a lesser percentage of slow digesting proteins (casein, 50% of total protein). Slow absorption is considered equally beneficial for providing a prolonged supply of AAs, which is particularly useful for muscle maintenance and growth over extended periods. P-ONS2 has a higher protein content (~11 g), resulting in greater bioavailability (AUC). TAAs, EAAs and BCAAs for both P-ONS1 and 2 reported a higher bioavailability (14% - 22% and 17% - 30% respectively) supporting a higher uptake in the circulation as compared to milk. With EAAs and BCAAs, being key regulators of protein anabolism showing much higher absorption into the serum and higher bioavailability along with a higher percent increase versus baseline levels as compared to milk, it underscores their beneficial impact on MPS.

Regarding individual AAs, methionine reported significantly higher absorption and bioavailability vs. milk for both ONSs. This is important as methionine is known for its antioxidant role, stabilizing the structure of proteins, recognition of protein surfaces, and as a regulatory switch through reversible redox reactions [32]. Similarly, tyrosine also showed significantly high Cmax and AUC for both P-ONS1 and 2. Tamura *et al.* in 2023 had demonstrated that Tyrosine is a key regulator of leucine-mediated protein synthesis [33] thus making tyrosine a potent regulator to increase MPS. P-ONS1 also exhibited a trend of elevated peak concentrations of histidine, with a p-value of 0.08 coming close to significance, marked by a 1-hour peak followed by a decline. Amino acids histidine and alanine have been observed to enhance anaerobic exercise capacity and performance through the multifunctional histidine containing dipeptide-carnosine (β-alanyl-l-histidine) [34] [35]. This suggests that regular consumption of P-ONS1 may enhance carnosine formation due to histidine, potentially alleviating fatigue in the elderly. It is well-established that the body can produce non-EAAs through endogenous synthesis, however a full range of EAAs and non-EAAs must be present in appropriate amounts for protein biosynthesis [36]. Therefore, the increase in concentrations of non-EAAs becomes equally important. Proline is known to play key roles in protein structure/function and maintenance of cellular redox homeostasis [37]. P-ONS1 showed significantly higher mean Cmax values for proline vs placebo, indicating improved absorption efficacy that could assist in homeostasis. Glycine is the simplest AA with two carbon atoms traditionally viewed as non-essential in mammals due to its endogenous synthesis. Glycine enhances muscle protein mass under an inflammatory condition [38] and has been shown as a novel nutritional intervention to restore the anabolic sensitivity of skeletal muscle to leucine with potential implications for a range of muscle-wasting conditions [39] which could occur in the elderly. P-ONS1 showed trends of higher absorption ($p = 0.06$) of this AA, which could prove beneficial for improving muscle protein mass in the elderly.

Fujita *et al.* reported that increases in MPS can be attributed to the postprandial rise in circulating EAAs, through inhibition of AMPK (AMP-activated Protein Kinase) and activation of the mTOR (mammalian target of rapamycin) signaling in human skeletal muscle, with an increase in protein synthesis [40], given this established relationship between EAA availability and MPS. Thus, the Cmax for 7 EAAs showing a swift increase ($p < 0.05$) in both P-ONS1 and P-ONS2 may be considered to have a positive impact on MPS. Additionally, animal proteins are more digestible and provide EAAs needed for MPS, particularly leucine. Owing to a combination of whey proteins faster digestion and absorption kinetics and higher leucine content, muscle protein accretion is more effective with whey protein as compared to casein or any other form of it [41] [42].

BCAAs are vital for MPS and muscle growth. They are unique because they are primarily metabolized in muscle tissue [43] [44] rather than the liver, providing a direct energy source during intense exercise and aiding in muscle recovery. Research suggests that BCAA supplementation may reduce exercise-induced muscle damage and fatigue, promoting better endurance and recovery [45]. Luiking *et al.* demonstrated that a low caloric leucine-enriched whey protein nutritional supplement provides a higher rise in serum levels of TAA, EAA and leucine compared to casein protein or high caloric products in healthy elderly subjects [46]. Hence, the whey and added leucine combination in P-ONS1 suggests a potential benefit in elderly populations. Both P-ONS1 and 2 showed significantly higher peak concentration levels of BCAAs when compared to milk. Research has shown that supplementing with BCAAs in low-protein diets (as prevalent in India), can enhance the net AA fluxes across skeletal muscle in vivo [47]. Both the products showed significant differences in absorption efficacy and bioavailability as compared to milk.

The stimulation of protein synthesis in the skeletal muscle is mediated through leucine via the mTOR pathway [48]. Rondanelli *et al.* observed that ingested free EAAs led to a faster increase in serum EAAs and faster disappearance from the blood as compared to dietary protein EAAs [49]; this finding is corroborated in this study as well. The added leucine in the P-ONS1 possibly assists in sustaining leucine levels in elderly individuals with reduced digestibility. Notably, leucine levels in P-ONS1 reached the highest mean serum concentration (315.6 nM/mL) at 30 minutes, possibly indicating faster absorption dynamic in play after which it maintained a plateau up to 90 minutes. P-ONS2 showed the highest mean serum concentration at 329.6 nM/mL at 30 minutes that decreased thereafter. Kamie *et al.* review observed that elderly individuals who consumed a leucine-rich supplement showed improvements in muscle mass, strength, and walking speed, while those who ingested BCAA also experienced enhanced MPS [17]. Hence, BCAAs can be beneficial for elderly individuals also looking to maintain or improve muscle mass and function. Additionally, BCAAs may aid in preserving muscle mass during periods of immobilization or bed rest, commonly experienced by older adults [50]. P-ONS1, with its added leucine content, has the potential to enhance MPS in the targeted elderly population.

Among the BCAAs, valine exhibited the highest serum concentration levels in this study. Valine metabolite, ß-aminoisobutyric acid (L-BAIBA) protects osteocytes from apoptosis, preventing bone loss [51] [52]. L-BAIBA is produced mainly in response to exercise, has beneficial effects on carbohydrate and lipid metabolism, mainly by enhancing insulin sensitivity, stimulating free fatty acid oxidation, and reducing proinflammatory cytokines [53]. Protein synthesis has been shown to occur in muscle fibres following their contraction that occurs during physical activity [54]. Physical activity is therefore key for muscle accretion to occur. It is observed the P-ONS2 demonstrates a significantly higher AUC (ie more bioavailable) than milk in the case of valine, while P-ONS1 versus milk does not show significant differences. A study by Liao *et al.* indicated that serum valine concentrations were significantly higher in Type 2 diabetes patients [55]. Further studies have stated that while leucine and milk-protein prevented high fat related adiposity [56], but valine supplementation and the ensuing high circulating serum concentrations, presented with insulin resistance, with no differential effects on insulin secretion during an OGTT [57]. Therefore, for an elderly population, P-ONS1 having highly bioavailable leucine and similar valine concentrations as compared to milk, proves to be a more favorable option, since the possibility of chronic conditions (diabetes, cardiovascular disease, hypertension) in the elderly is higher. P-ONS2 is a more ideal supplement for a younger and more active population with no chronic conditions.

In essence, the study suggests that both P-ONS1 & P-ONS2 will effectively address any deficits in AAs to enhance muscle health without overloading the body with excessive proteins, that could have adverse effects. Church *et al.* demonstrated in their study that a 100% increase in peripheral (extracellular) EAA concentrations increased fractional synthesis rate (FSR; uptake into the cell) by ~34% [58], the study concluded that a large variety of EAA/protein-containing formats and food as well as large increases in peripheral EAA concentrations are required to drive a robust increase in muscle and whole-body protein synthesis. P-ONS1 and 2 when consumed as powders (30 g) added to water, provide 30% - 49% of the EAR of AA (mg/kg/d) for 8 of the 9 EAAs analyzed. When consumed in milk as done in this study, it meets >50% - 100% of EAR across different EAA. For adults following predominantly plant-based diets, these ONSs can help meet their EAA needs, which might otherwise be low or even lacking in the diet.

The study offered valuable insights into the acute effects of the P-ONSs suggesting improved absorption and bioavailability of AAs. However, as this was an acute study it has the limitation of not being able to provide the impact of these P-ONSs post long-term consumption. Additionally, as this study was primarily an absorption study (pharmacokinetics), it involved healthy volunteers. Thus, the role and impact of these P-ONSs in different target population groups (like active adults/elderly/people with chronic conditions) needs further evaluation. The efficacy of these protein supplements on muscle strength, physical function and mobility in different population groups through long-term (≥6 months) supplementation trials will be helpful.

5. Conclusion

The study has shown that both P-ONS1 and P-ONS2 provide a substantial pool of AAs in serum within minutes of consumption. Long-term consumption can improve protein quality and/quantity of Indian diets, which could further support muscle health.

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Authors Contributions

D.K., H.S.G., M.K., J.B., were primarily involved in the design and conceptualization of the study. M.K., and S.K.M., were involved in data collection, study conduct, protocol execution, quality checks, and data verification. S.T.R., was responsible for the statistical analysis of results. M.S.S., was involved in the product amino acid analysis. D.K., and J.B. were involved in operational decision making during the conduct of the study. S.K.M., S.T.R., and M.K., interpreted the study results and participated in data curation. D.K., M.K., and J.B., were overall responsible for the conduct of the study ethically and scientifically. H.S.G. drafted and led the manuscript for its intellectual content, literature searches, and graph generation. H.S.G. and D.K. jointly led the manuscript creation and carried out interpretations regarding intellectual content. The final version of the manuscript was carefully reviewed and approved by all authors. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

The study was performed in accordance with the protocol, Good Clinical Practice (GCP) guidelines, local regulations governing clinical conduct, and the ethical principles that have their origin in the Declaration of Helsinki. The study protocol was approved by the Good Society Ethical Research dated 11th December,2023, protocol code (NUN-HFD-006/23 Version 1.0). The study objectives were explained to all participants, who voluntarily gave written informed consent prior to enrolment. The study was registered with the Clinical Trials Registry-India (CTRI/2023/12/060650 (Available at CTRI https://ctri.nic.in/Clinicaltrials/pmaindet2.php?EncHid=OTY2MDI=&Enc=&userName=).

Informed Consent Statement

The informed consent form was reviewed and approved by the Institutional Ethics Committee of "Good Society Ethical Research" on 11th December 2023. All participants voluntarily gave written informed consent prior to enrolment.

Data Availability Statement

Ethical restrictions imposed by the IEC prevent public sharing of the data for this study. The data used in this publication is owned by HUL (Foods). Data access requests will be evaluated by HUL (Foods) in consideration of IEC requirements. Interested researchers will need to sign a research collaboration agreement with HUL (Foods). Requests can be sent to D.K.

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Conflicts of Interest

D.K., H.S.G., J.B., and M.S.S., declare potential conflicts of interest as employees of Hindustan Unilever Limited (HUL), the study sponsor. All other authors declared no potential conflict of interest.

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Supplementary Information

Box S1. Analytical analysis of ONS amino acid content.

SAMPLE PREPARATION	Sample preparation was performed using both acid hydrolysis as well as alkaline hydrolysis. Acid hydrolysis method was used for quantification of 17 amino acids (except tryptophan) whereas for tryptophan alkaline hydrolysis method was used.
AMINO ACID ANALYSIS	• Amino acid analysis was performed using 5 levels (Range: 0.001 mM - 0.050 mM concentration) of calibration standards (Amino acids standard, Hydrolysate (Waters, Part No. WAT 088122 or equivalent), containing Aspartic acid, Serine, Glutamic acid, Glycine, Histidine, Arginine, Threonine, Alanine, Proline, Cystine, Tyrosine, Lysine, Valine, Methionine, Isoleucine, Leucine and Phenyl Alanine. Tryptophan (Sigma product code T0254 or equivalent). Norvaline (Sigma product code N7627 or equivalent) was used as internal standard. • The extraction and derivatization of amino acids to their corresponding N-ethoxy carbonyl ethyl ester derivative was performed using Waters AccQ-Tag solutions and reagents. • For 17 amino acids (except tryptophan) 10.0 mg of sample was subjected to acid hydrolysis (20 mM of HCl with 2% Phenol) for 22 hours in hot air oven maintained at 110˚C ± 5˚C for 17 amino acids. • Acidified sample was then neutralized before derivatization. For tryptophan, 10 mg of sample was hydrolyzed for 20 hours in hot air oven maintained at 110˚C ± 5˚C using sodium hydroxide solution (4 N solution). The derivatized solution (0.2 µl) was injected in HPLC -FLD system (Agilent UHPLC 1290 or Waters H-Class ACQUITY UPLC). • Amino acids were separated on AccQ-Tag, Ultra C18 1.7 µm, 2.1 × 100 mm column, Waters, Part No.: 186003837) with column inline filter (Waters Part No.:205000343) maintained at 55˚C, with a flow rate of 0.7 ml/min. LC-Column, (Agilent Eclipse plus RRHD, 1.8 µm, 2.1 × 50 mm, Part No.: 959757-902) was used for Tryptophan analysis. A 12.5 minute gradient elution program was used for elution of 17 amino acids with aqueous mobile phase A containing 25 ml of AccQ-Tag Eluent A concentrate (Waters, Part No: 186003838) into a standard 25 ml volumetric flask and transfer accurately into 475 ml of Milli Q water and mobile phase B containing 6.556 ml of Formic acid (MS grade) to 500 ml Acetonitrile. • The gradient elution program was started with 0.1% B (99.9% A), which was increased to 2% (98% A) over 3 min. By 9.0 min, the proportion of B was increased to 90% (10% A) and was finally it was brought back to the initial concentration of 0.1% B (99.9% A), with a hold time of 1.0 min. For elution of tryptophan isocratic elution was performed using acetate buffer pH 5.0 at a flow rate of 0.2 ml/min.
QUALITY CONTROL	Quality control sample was prepared using reference product of known amino acid concentration with an acceptance criterion of 80% - 110% recovery. Batch sequence and quality control acceptance criteria for analysing amino acids in the products consisted of solvent blank (analyte free) followed by calibration standards ($R^2 \geq 0.995$), reagent blank (analyte free), QC sample (80% - 110% recovery), sample under study and finally calibration check standard (80% - 110% recovery). The method precision *i.e.*, repeatability and reproducibility established for the test method was <5% and <8%, respectively.

Table S1. Percent estimated average requirements (% EAR) of essential amino acids delivered by ONS 1 and ONS 2 (in milk).

	Essential Amino Acids*	mg/kg/d*	Average adult requirements (60 kg)	ONS1$ (mg/30g)	ONS2$ (mg/30g)	%EAR ONS1 + milk	%EAR ONS2 + milk
1.	Histidine	10	600	171	264	52	67
2.	Isoleucine	20	1200	360	525	63	77
3.	Leucine	39	2340	924	837	69	66
4.	Lysine	30	1800	843	936	78	83
5.	Methionine	10	600	186	699	58	143
6.	Threonine	15	900	591	738	101	117
7.	Phenylalanine + Tyrosine	25	1500	357	393	46#	49#
8.	Tryptophan	4	240	36	6	57	44
9.	Valine	26	1560	405	525	53	61

*Column data adapted from RDA ICMR 2020; $Values from in-vitro analyses of ONS1 and ONS2; #EAR % is for phenylalanine alone.

Table S2. Cmax (Mean ± SE)/absorption efficacy for all amino acids analysed for both ONS1 and ONS2 versus placebo (milk).

Amino acid (nM/ml)		ONS1	ONS2	Placebo	P value Cmax ONS1 vs placebo	P value Cmax ONS2 vs placebo
Essential Amino acids						
1.	Histidine	179.5 ± 58.7	167.7 ± 48.7	152.6 ± 42.5	0.0795^	0.2631
2.	Lysine	301.1 ± 69.9	329.1 ± 74.21	251.7 ± 73.1	0.0226*	0.0007**
3.	Methionine	55.8 ± 9.3	61.2 ± 13.5	45.0 ± 15.90	0.0047*	0.0003**
4.	Phenylalanine	203.1 ± 45.5	201.2 ± 36.1	174.5 ± 45.9	0.0371*	0.0300*
5.	Threonine	251.5 ± 63.9	247.4 ± 57.1	190.1 ± 55.1	0.0007*	0.0007**
6.	Isoleucine	334.7 ± 92.4	338.3 ± 80.8	251.9 ± 85.6	0.0007**	0.0007**
7.	Leucine	315.6 ± 78.0	329.6 ± 79.3	241.1 ± 78.0	0.0007**	0.0007**
8.	Valine	422 ± 126.4	448.9 ± 166.6	343.9 ± 114.7	0.0307*	0.0141*
Conditional and non-essential amino acids						
1.	Alanine	714.3 ± 129.5	701.0 ± 161.0	655.2 ± 162.5	0.1761	0.3324
2.	Arginine	301.9 ± 109.7	296.7 ± 113.2	262.0 ± 124.3	0.2500	0.3200
3.	Aspartic acid	59.7 ± 36.4	52.0 ± 21.9	48.9 ± 20.7	0.2148	0.6105
4.	Cysteine	88.9 ± 60.3	84.0 ± 61.7	68.2 ± 53.5	0.2200	0.3500
5.	Glutamic acid	334.4 ± 109.8	323.3 ± 125.4	292.9 ± 87.0	0.1572	0.3345
6.	Glycine	521.7 ± 142.4	455.9 ± 116.0	449.1 ± 113.3	0.0588^	0.8372
7.	Proline	498.1 ± 125.6	490.3 ± 157.7	416.8 ± 147.3	0.0482*	0.1022
8.	Serine	347.2 ± 251.5	264.8 ± 97.7	250.7 ± 84.9	0.0822^	0.5955
9.	Tyrosine	149.6 ± 44.0	145.6 ± 2.5	117.9 ± 41.1	0.0130*	0.0128*

*significant at $p < 0.05$; highly significant at $^{**}p < 0.001$; ^strong trends but marginally missed significance.

Table S3. AUC (Mean ± SE)/Bioavailability efficacy for all amino acids analysed for both ONS1 and ONS2 versus placebo (milk).

Amino acid (nM/ml. h)		ONS1	ONS2	Placebo	P value ONS1 vs placebo	P value ONS2 vs placebo
Essential Amino acids						
1.	Histidine	209.3 ± 55.4	203.9 ± 57.7	187.1 ± 49.3	0.1536	0.2853
2.	Lysine	350.4 ± 70.6	388.6 ± 90.5	308.4 ± 100.9	0.1065	0.0057*
3.	Methionine	66.7 ± 10.5	75.6 ± 16.4	56.0 ± 18.7	0.0202*	0.0003**
4.	Phenylalanine	239.0 ± 46.7	245.2 ± 51.20	216.4 ± 59.2	0.1540	0.0780^
5.	Threonine	293.4 ± 69.1	294.6 ± 68.7	233.9 ± 66.2	0.0007**	0.0007**
6.	Isoleucine	392.0 ± 100.6	407.9 ± 99.6	301.3 ± 110.9	0.0053**	0.0007**
7.	Leucine	368.1 ± 98.0	394.7 ± 91.7	290.6 ± 97.8	0.0053**	0.0007**
8.	Valine	489.4 ± 137.3	528.9 ± 172.1	430.0 ± 140.9	0.1504	0.0346*

Continued

Conditional and Non-essential Amino acids						
1.	Alanine	880.0 ± 181.3	893.9 ± 198.6	818.8 ± 210.5	0.2920	0.1761
2.	Arginine	343.3 ± 122.9	355.4 ± 127.4	315.5 ± 142.3	0.4791	0.3115
3.	Aspartic acid	55.4 ± 23.3	54.8 ± 18.6	49.9 ± 22.0	0.4124	0.4095
4.	Cysteine	104.1 ± 71.1	102.1 ± 74.6	82.4 ± 64.6	0.2800	0.3300
5.	Glutamic acid	387.5 ± 107.5	400.8 ± 160.9	367.7 ± 108.9	0.5352	0.4092
6.	Glycine	596.4 ± 119.2	569.0 ± 131.8	546.8 ± 132.4	0.1851	0.5630
7.	Proline	588.1 ± 136.0	607.9 ± 184.7	514.1 ± 176.6	0.1157	0.0787^
8.	Serine	344.9 ± 139.7	316.0 ± 96.2	291.9 ± 98.4	0.1383	0.3959
9.	Tyrosine	168.2 ± 39.8	175.7 ± 40.6	141.2 ± 47.7	0.0413*	0.0099*

*Significant at $p < 0.05$; highly significant at **$p < 0.001$; ^strong trends but marginally missed significance.