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Metabolomics and gut metagenomics profile of the healthy adults on consumption of whey protein supplemented with enzymes-probiotics blend

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Abstract
In this randomized, crossover, pilot clinical study, we aimed to evaluate the effect of supplementation of enzymes-probiotics blend with whey protein on the amino acid absorption and gut microbiota. Healthy subjects were supplemented with the whey protein and test i.e. Pepzyme Pro (enzymes-probiotics blend) or placebo i.e. maltodextrin for 15 days with the washout period of 30 days. Blood samples were analyzed for plasma free amino acids, insulin, and CRP. Additionally, urine nitrogen, fecal nitrogen, and gut microbiota were evaluated. On day 15, the test arm showed upward trend in rate of amino acid absorption than placebo arm within 30 min of post ingestion of protein. Moreover, rate of absorption of few essential and branched chain amino acids were significantly higher (methionine ($p = 0.049$), leucine ($p = 0.014$), isoleucine ($p = 0.053$)) in the test arm on day 15. Total branched chain amino acids absorption were found to be significantly higher ($p \leq 0.05$) in the test arm than the placebo arm within 30 min of post ingestion on day 15. Uptrend in total amino acid absorption and C_{max}, and downtrend in T_{max} was observed on day 15 in the test arm. The CRP, fecal nitrogen, and urine nitrogen remained unaltered after supplementation. Microbiota profiling showed significant change in abundance of species of genus *Bacteroides* and phylum *Bacteroidetes*. Overall, metagenomics and metabolomics based assessments demonstrated that the consumption of Pepzyme Pro with whey protein could potentially improve protein digestion, amino acid absorption, and modulate gut microbiota.
Clinical trial registration The clinical trial registry of India CTRI/2021/09/036169 [Registered on: 02/09/2021]

Article Highlights

- Uptrend in the rate of amino acid absorption and total amino acid absorption after consumption of whey protein with enzymes-probiotics blend for 15 days.
- Significantly higher rate of absorption of branched chain amino acids after consumption of whey protein with enzymes-probiotics blend for 15 days.
- Positive alteration in gut microbiota after consumption of whey protein with enzymes-probiotics blend for 15 days.

Keywords Digestion · Absorption · Protein · Pepzyme Pro · Proteases · Probiotics · Amino acids

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1 Introduction

Consumption of an adequate amount of dietary protein is an absolute requirement to maintain the optimal health, growth, development, and function of the body. It maintains skeletal muscle mass crucial for healthy and quality life. The quality and quantity of protein ingested, its absorption kinetics, and the digestibility are key factors contributing to the benefits of the protein [1, 2]. Protein and amino acid supplements are widely available in the market and consumed majorly by athletes and recreationally active adults. These supplements are imperative in sport nutrition, medical foods, muscle recovery products, satiety and weight management foods, and geriatric products [3].

Whey protein is a high quality popular protein supplement rich in essential amino acids [4]. Consumption of whey protein after intensive eccentric exercise has been reported to reduce the efflux of muscle damage biomarkers [5]. The efficacy and safety of whey protein supplement as an ergogenic aid on athlete's sports performance and recovery are thoroughly documented in the literature [6]. A growing body of research indicates that protein intake well above the recommended daily allowance (RDA, 0.8 g/kg/d) helps to promote appetite regulation, weight management, and goals aligned with athletic performance [7]. The rate of digestion of protein purportedly linked to muscle protein synthesis. However, these benefits cannot be obtained without optimum digestion and absorption of the consumed protein [8]. In fact, the digestibility of the whey protein gets affected by the industrial processing that reduces its therapeutic effects. Incompletely digested protein reaches the colon, causes detrimental effects that negatively affect the gut microflora and hence the overall health [9–11]. Incomplete protein digestion changes the ratio of *Bacteroidetes* to *Firmicutes* in the gut which is associated with increased tumorigenesis and colorectal cancer. It further leads to the reduction of carbohydrate-utilizing microbiota such as *Lachnospiraceae, Ruminococcaceae, Prevotella, Bifidobacterium animalis, Faecalibacterium prausnitzii,* and *Ruminococcus bromii* [12]. A high protein diet taken for a long time has been shown to decrease beneficial organisms in human gut microbiota, especially propionate-producing and butyrate-producing bacteria. A low level of propionate and butyrate in the gut creates a conducive environment for the growth of pathogenic bacteria [13].

The protein digestion can be improved by consuming hydrolysate instead of whole protein. Though protein hydrolysate showed the accelerated protein digestion and absorption in the gut [14], consuming protein hydrolysate has been found to impair the proteases secretion by the digestive system [15] on the other hand. Hence, the interest is to improve the digestion and absorption of the consumed protein without affecting the body's natural digestive processes. The supplementation of protease and/or probiotic in the protein is of particular interest. Few clinical studies have demonstrated that the digestion and absorption of the protein were improved by blending the protein with enzymes [10, 16, 17] or probiotics [18]. Further, the supplementation of enzymes (phytase and xylanase) and probiotics in *Nile tilapia* demonstrated increased nutrient availability and stabilized the microbiome environment that benefits the gut health [19]. However, the study of the microbiome modulation after consuming protein with either enzymes and/or probiotics have not yet been reported.

With this background it was speculated that the blend of enzymes and probiotics would add value in the protein digestion and would alter the microbiota positively imparting gut health. Pepzyme Pro is a commercial formulation of a blend of enzymes (proteases from *Aspergillus niger*) and probiotics (*Bacillus coagulans, Bacillus clausii, Bacillus subtilis, Lactobacillus plantarum,* and *Lactobacillus acidophilus*). Our earlier study on the protein digestion under simulated in-vitro gastrointestinal conditions have demonstrated the ability of the enzymes blend to improve the digestion of the protein (whey, pea, and collagen) as well as the release the bioactive peptides [20]. The current pilot clinical study was designed to determine the effect of enzymes-probiotics blend on the whey protein digestion and absorption. The effect of the supplement on the gut microbiota was also in the scope of this study.

2 Materials and methods

2.1 Formulation/investigational product (IP)

Pepzyme Pro was the investigational product (IP) supplied by Specialty Enzymes and Probiotics, Chino, USA. It contained the blend of proteolytic enzymes (Proteases from *Aspergillus niger*) and probiotics (*Bacillus coagulans* LBSC DSM (17654), *Bacillus clausii* 088AE (MCC 0538), *Bacillus subtilis* PLSSC (ATCCSD 7280), *Lactobacillus acidophilus* 033AE, and

Lactobacillus plantarum 022AE (MCC 0537). The maltodextrin (excipient) was used as a placebo. The test product was whey protein (30 g) along with Pepzyme Pro (1% of the protein) and the placebo product was whey protein (30 g) along with maltodextrin (1% of the protein). Both test and placebo products complied with the specification. The packaging and labelling for both the products were same, except for the coded batch numbers used for differentiation. The participants, the investigators, and the study team were blinded to the treatment allocation.

2.2 Ethics and informed consent

This pilot clinical study was approved by Institutional ethics committee of Charak Hospital (Registration No.: ECR/1562/Inst/MP/2021), Bhopal, Madhya Pradesh, India and registered with Clinical Trial Registry India (CTRI) (CTIR No.: CTRI/2021/09/036169) on November 02, 2021. The study was conducted in conformity with ICH-GCP (E6 R2) guidelines, the Helsinki Declaration, and the local regulatory requirements (Indian GCP, Indian Council of Medical Research, and New Drugs and Clinical Trials Rules-2019). The approved protocol was followed with no further changes or amendments during the trial. All subjects were provided with the complete information about the study in written, visual, and oral form in an understandable language. Every subject had given the digital informed consent to the investigator after understanding the objective of this trial, including possible risks and benefits. Based on the data available for safety and adverse events, the employed study design, duration of treatment, and medical surveillance during the study; there seems to be no unacceptably high risk to the subjects. Both the sponsor and the investigator consider the trial to be ethically acceptable.

2.3 Study design and selection of study subjects

This prospective interventional trial was a randomized, double-blinded, placebo controlled, and crossover study and had six visits to the clinical site by each study subject. Subject selection was done as per the following inclusion and exclusion criteria.

2.3.1 Inclusion criteria

Adult Indian male or female with age ≥ 18 and ≤ 35 years; normal body weight (body mass index (BMI) of 19–24.99 kg/m^2); willingness to provide written informed consent and comply with study instructions for its duration; must be of normal health as determined by medical history and physical examination; screening laboratory values are within normal limits. Participants who met the necessary inclusion criteria were further encouraged not to change their current physical activity levels and to refrain from exercise for 24 h before starting the clinical trial.

2.3.2 Exclusion criteria

Subjects with organ transplantation or surgery in the past 6 months; known hypersensitivity or idiosyncratic reaction or intolerance to protein diet or any ingredients of the formulation or any related products as well as severe hypersensitivity reactions (like angioedema) to any drugs or food products; women subjects that are pregnant or lactating; history of smoking or tobacco consumption; history of clinically significant, cardiovascular, pulmonary, hepatic, renal, hematological, gastrointestinal, endocrine, immunologic, dermatologic, musculoskeletal, neurological, or psychiatric disease; difficulty with donating blood.

2.3.3 Randomization, study protocol, and supplementation

The current randomized crossover pilot clinical study was carried out at Bhopal, India. It consisted of screening and baseline phases followed by two supplementation phases that each spanned 15 days and were separated by a washout period of 30 days. A total of 15 male subjects were randomized (block randomization was done on the online randomization tool www.randomization.com using pseudo number generator) to either the test arm that received whey protein and IP or the placebo arm that received a whey protein and placebo for 15 days. In the supplementation period (day 1 to 15), all the participants were instructed to take a test or placebo supplement along with 300–500 ml of luck warm water on empty stomach daily in the morning. Daily diet was recorded by the subjects. The occurrence of adverse events was recorded throughout the study. On day 1 and 15 of each supplementation period site visit was planned for each subject. Upon visit, subjects were instructed to ingest respective supplement and blood samples were taken at 0, 30, 60, 120,

180, and 240 min post ingestion and analysed for the plasma free amino acids. For insulin and C-reactive protein (CRP) blood sampling was done on day 15 (0 h and 4 h) post ingestion of the supplement at both the supplementation cycle. Faecal and urine (24 h) samples were collected on Day 1 and 15 on each supplementation period and analysed for nitrogen content. The faecal samples of all the test subjects collected on the Day 1 and Day 15 (of both the supplementation period) were analysed for the gut microbiota.

2.4 Analysis

Plasma free amino acids were analyzed at Thyrocare Mumbai, India using liquid chromatography tandem mass spectrometry method. Insulin and CRP were analysed at Thyrocare Mumbai, India by fully automated Electrochemiluminescence Immunoassay and Immunoturbidimetry, respectively. Urine and fecal nitrogen content were analysed by Dumas combustion method. Gut microbiota was analysed by Leucine Rich Bio Pvt Ltd. India using shotgun microbiome sequencing method as mentioned below:

2.4.1 Sample collection

Stool samples were collected from all the test subjects using Invitek Molecular Stool Collection Module (Cat. No. 1038111300, Berlin, Invitek Molecular GmbH). The stool collection tube contained DNA stabilizing solution (8 ml) and an integrated spoon in cap. Subjects were instructed to collect ~ 4–5 spoons of stool into the stabilizing solution. Once collected, they were instructed to gently mix the sample for 15 s, seal and then shipped under room temperature to the processing unit for DNA extraction.

2.4.2 DNA extraction

DNA was extracted from stool samples using QIAamp® Fast DNA Stool Mini (Cat No./ID: 51604, QIAGEN) following the manufacturer's "Fast DNA Stool Mini Handbook" for fast purification of genomic DNA. Eluted DNA was collected in 1.5 ml DNA LoBind microcentrifuge tubes, and the quantity and quality were assessed by Qubit 2.0 DNA HS Assay (ThermoFisher, Massachusetts, USA) and NanoDrop® (Roche, USA) to meet the sequencing requirements.

2.4.3 Sequencing and analysis

Whole metagenome sequencing was performed on all the samples using long read sequencing technology. Raw sequencing reads were stored in FastQ format for further computational analysis. The upstream analysis involved quality check and quality improvement measures, including but not limited to host (human) sequence removal. This was followed by alignment of quality processed reads to a reference database of microbial genomes. The raw and % normalized abundances, of all the microorganisms identified within these samples, were quantified, and later used for downstream analysis involving various statistical measures. Taxonomic composition of communities across samples and comparing groups were visualized for direct quantitative comparison of abundances. Single factor ANOVA was performed to estimate the variance and its statistical significance across the comparing groups as well as for all the kingdoms and phylum. Percentage bar plots were created for comparing group, Day 1 (Whey Protein_Pre) vs Day 15 (Whey Protein_Post), for viewing the composition at various taxonomic levels. Alpha diversity was characterized using different measures such as Chao1 index and Shannon index. Non-phylogenetic beta diversity analysis was performed employing Bray–Curtis distance. Principle Coordinate Analysis (PCoA) was used to visualize the distance matrix created by the beta diversity analysis and statistical significance of the clustering pattern in PCoA plots were evaluated using Permutational ANOVA (PERMANOVA). Differential abundance (DA) analysis was done using five different DA tools, viz., Univariate Analysis, metagenomeSeq, EdgeR (v3.12), DeSeq2, and LEfSe (Linear discriminant analysis Effect Size) to identify those microbial species that were called significantly ($p < 0.01$) differentially abundant in "consensus" by all five DA tools, ensuring the robustness of the DA characterization.

2.5 Efficacy and safety variables

Primary endpoints were set to study the effect of dietary supplement Pepzyme Pro on the digestion and absorption of whey protein. It included the assessment of change in plasma free amino acids within 4 h of consumption of protein

in the placebo arm and test arm and pairwise comparison between both the arms. Secondary endpoints were set for the evaluation of change in nitrogen level in fecal sample and urine sample, change in serum insulin and CRP level, and change in gut microbiome in fecal sample. Secondary endpoints also included safety evaluation by the assessment of adverse events, vital signs (pulse rate, systolic and diastolic blood pressure (seated)), body temperature, respiratory rate, and physical examination.

2.6 Sample power and statistical analysis

Data was analysed with 5% significance level (confidence interval 95%) and maintaining a minimum power of 80% for study using SAS software, version 9.1. Separate analyses were performed for primary and secondary endpoints. Linear trapezoidal rule was used to calculate the area under the concentration *vs*. time curve (AUC) for each amino acid at all available time points. The comparison between the test and placebo arm were done using student's *t* test. Data was represented as mean ± standard error (SE) unless specified. p value ≤ 0.05 was considered statistically significant unless specified.

3 Results

The current pilot clinical study was initiated on October 17, 2021 and completed on January 8, 2022. 15 healthy male subjects between age 18–35 years participated in the study (Fig. 1). The demographic details of the subjects at the baseline are presented in Table 1. Principal investigator and clinical trial team assessed study regulations at each visit along with all the safety and efficacy assays as per the schedule of events (Table 2). The clinical trial was concluded after the completion of target sample size and follow-up visit of the last enrolled patient according to the study procedures.

3.1 Primary endpoint: efficacy evaluation

3.1.1 Change in rate of absorption of plasma free amino acids after consumption of protein

Change in plasma free amino acids on consumption of test or placebo supplement was assessed on day 1 and day 15. On both the days, test arm showed upward trend in the rate of absorption of amino acids over the placebo arm. On day 1, alanine (2.095 ± 0.864 µmol/min, placebo and 5.170 ± 3.410 µmol/min, test), glutamine (1.518 ± 1.223 µmol/min, placebo and 6.333 ± 4.469 µmol/min, test), proline (1.157 ± 0.524 µmol/min, placebo and 2.559 ± 1.162 µmol/min, test), and serine (0.537 ± 0.172 µmol/min, placebo and 1.155 ± 0.555 µmol/min, test) had shown the propensity to increase the rate of absorption (Fig. 2A). On day 15, alanine (0.848 ± 1.353 µmol/min, placebo and 2.272 ± 1.170 µmol/min, test), glutamine (1.595 ± 1.127 µmol/min, placebo and 4.153 ± 1.180 µmol/min, test), isoleucine (1.254 ± 0.396 µmol/min, placebo and 2.512 ± 0.481 µmol/min, test), leucine (1.190 ± 0.485 µmol/min, placebo and 3.102 ± 0.550 µmol/min, test), methionine (0.186 ± 0.056 µmol/min, placebo and 0.403 ± 0.090 µmol/min, test), phenylalanine (0.104 ± 0.073 µmol/min, placebo and 0.288 ± 0.081 µmol/min, test), serine (0.219 ± 0.090 µmol/min, placebo and 0.591 ± 0.192 µmol/min, test), and tyrosine (0.193 ± 0.144 µmol/min, placebo and 0.489 ± 0.126 µmol/min, test) had shown tendency to increase the rate of absorption (Fig. 2B). The rate of absorption of amino acids in test arm on day 1 was not statistically different than placebo but, on day 15, the rate of absorption of the isoleucine ($p = 0.053$), leucine ($p = 0.015$), and methionine ($p = 0.049$) showed statistically significant increase in the test arm compared to the placebo arm.

Increase in the essential amino acid concentration within the 30 min of post ingestion of the protein showed upward trend in the test arm compared to the placebo arm on both day 1 and day 15. Although all the branched chain amino acids were in uptrend in the test arm than the placebo arm on both the day of analysis; isoleucine ($p = 0.053$) and leucine ($p = 0.015$) had shown statistically significant difference between the test and placebo arm on day 15 (Fig. 3). Increase in total branched chain amino acid (TBCAA) concentration within 30 min was statistically higher in the test arm than the placebo arm ($p = 0.032$).

3.1.2 Absorption of total plasma free amino acids after consumption of protein

Absorption of total free amino acids was assessed in terms of area under concentration (AUC), C_{max} and T_{max}. AUC is total free amino acids present in the blood within 4 h of consumption of the protein. C_{max} is a maximum observed

Fig. 1 Participant flow chart

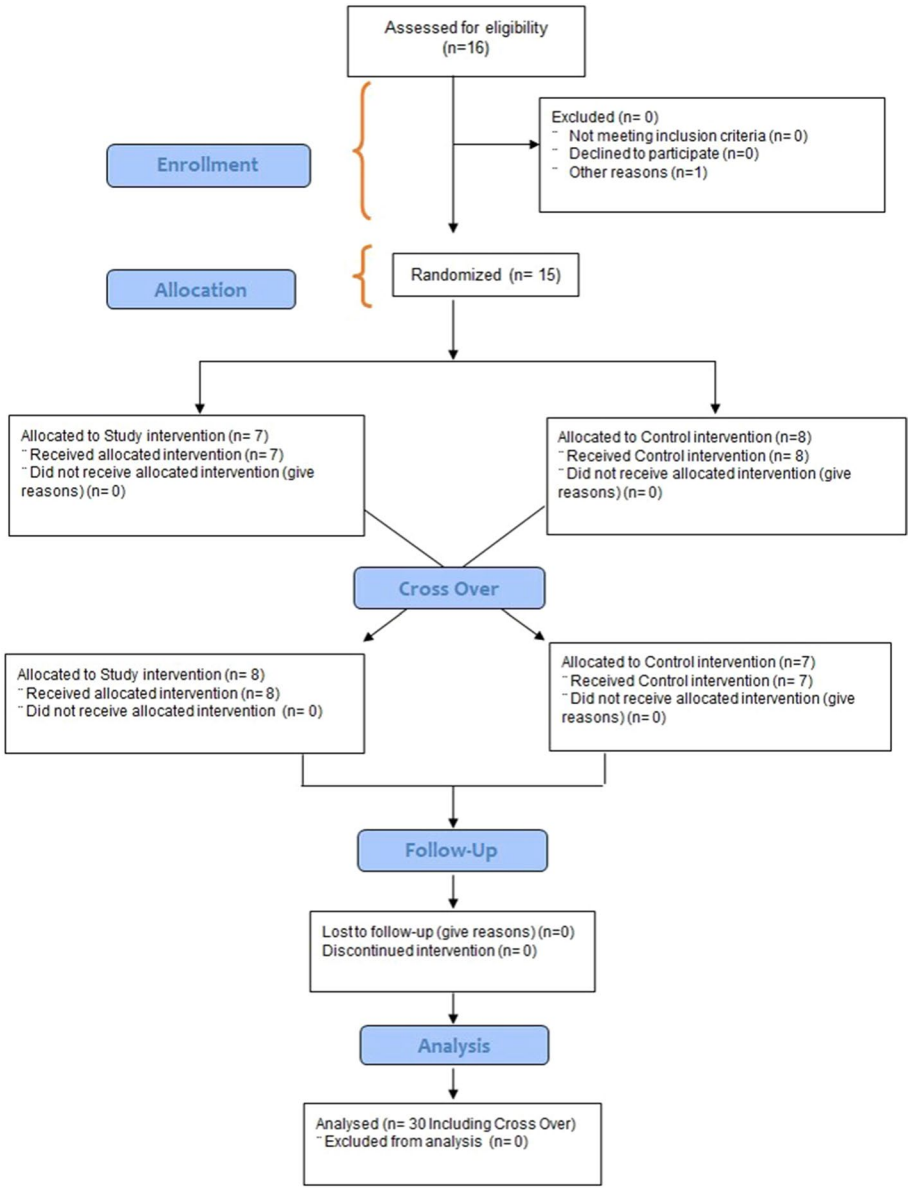

Table 1 The baseline characteristics of study subjects

Characteristics	Total (n = 15)
Gender	Male
Age (Years)	24.7 ± 3.5
Height (cm)	173.2 ± 7.2
Weight (kg)	66.9 ± 7.9
BMI (kg/m^2)	22.3 ± 2

BMI: Body mass index, Values represented as mean ± standard deviation

concentration and T_{max} is the time at which C_{max} is reached. On day 1, AUC and C_{max} for glycine (AUC 8.98%, C_{max} 11%), methionine (AUC 12.27%, C_{max} 13.70%), serine (AUC 10.46%, C_{max} 14.97%), threonine (AUC 11.15%, C_{max} 15.02%), tryptophan (AUC 19.55%, C_{max} 24.03%), tyrosine (AUC 11.99%, C_{max} 15.9%), and valine (AUC 13.65%, C_{max} 15.69%) were in a upward trend in the test arm compared to that of in the placebo arm (Table 3). Similarly, on day 15, test arm showed upward trend in absorption of arginine (AUC 22.26%, C_{max} 15.68%), asparagine (AUC 20.58%, C_{max} 19.19%), isoleucine (AUC 20.92%, C_{max} 27.55%), lysine (AUC 16.04%, C_{max} 13.85%), methionine (AUC 17.9%, C_{max} 18.63%), serine (AUC 13.86%,

Table 2 Study design

Assessment	Screening		Treatment period			Washout	Treatment period			Safety follow-up
		Baseline testing	Day 1	Day 2–14	Day 15	Day 16–30	Day 31	Day 32–44	Day 45	Day 46–60
Visit Site (*Overnight fasting)	X	X	X*		X*		X*		X*	
Written Informed Consent	X									
Inclusion/Exclusion criteria	X		X							
Randomization			X							
Demographics	X									
Body height and weight	X	X	X		X		X		X	
Medical/surgical history	X									
Prior medication history	X									
Physical examination	X		X	X	X	X	X	X	X	X
Vital signs	X	X								X
Instructions for supplementation	X	X								
Instructions for dietary recording	X	X								
Dietary restriction	X	X								
Dietary record check			X		X		X		X	
Dietary recording				X		X		X		
Study drug administration (With/Without Supplement)			X	X	X		X	X	X	
Blood sampling assessment (0 h, 0.5 h, 1 h, 2 h, 3 h, 4 h)			X		X		X		X	
Blood analysis 0 and 4 h sampling (CRP and Insulin)					X				X	
Urine Analysis (24 h urine collection)			X		X		X		X	
Faecal Sample Analysis (faecal Nitrogen analysis)			X		X		X		X	
Gut microbiome analysis			X		X		X		X	
Adverse event recording	X	X	X	X	X	X	X	X	X	X
Concomitant medication review	X	X	X	X	X	X	X	X	X	X

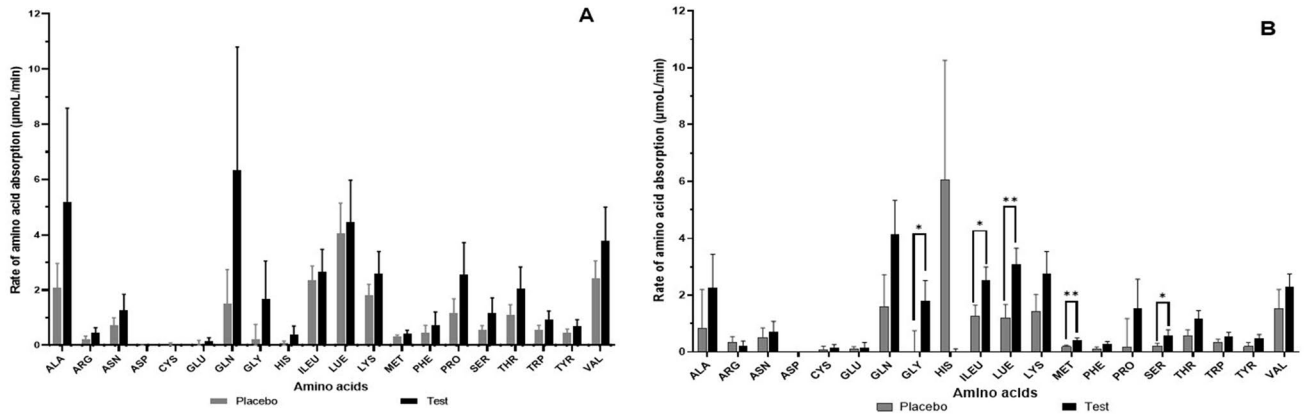

Fig. 2 Rate of amino acid absorption (µmoL/min) within 30 min of consumption of whey protein + maltodextrin (placebo) *Vs* whey protein + Pepzyme Pro (test) on day 1 (**A**) and day 15 (**B**). Values represented as mean ± standard error. ** and * represent significant difference between placebo and test arm at $p \leq 0.05$ and $p \leq 0.1$ respectively

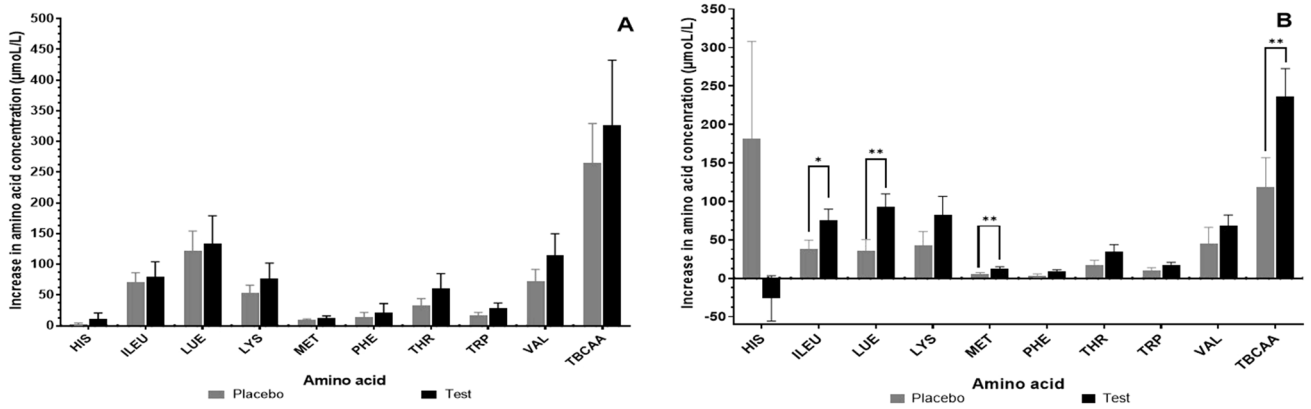

Fig. 3 Increase in essential amino acid (EAA) and total branched chain amino acid (TBCAA) concentration (µmoL/L) within 30 min of consumption of whey protein + maltodextrin (placebo) *Vs* whey protein + Pepzyme Pro (test) on day 1 (**A**) and day 15 (**B**).Values represented as mean ± standard error. ** and * represent significant difference between placebo and test arm at $p \leq 0.05$ and $p \leq 0.1$ respectively

C_{max} 15.19%), threonine (AUC 18.56%, C_{max} 16.23%), and tryptophan (AUC 12.14%, C_{max} 11.67%) compared to the placebo arm (Table 4). Although T_{max} was found to be unaltered on day 1 (Table 5), it showed tendency to decrease in the test arm compared to the placebo arm on day 15. Specifically T_{max} of the glycine ($p = 0.016$), methionine ($p = 0.018$), and valine ($p = 0.029$) showed statistically significant difference among the placebo and test arm (Table 6).

3.2 Secondary endpoints

During the course of study, no adverse effects were reported by any of the subjects, indicating safety of the supplements.

3.2.1 Change in nitrogen level in fecal sample and urine sample

Nitrogen level in the fecal and urine samples were analysed for all the subjects on day 1 and day 15. The results showed that there was no statistically significant difference between the nitrogen level in the fecal and urine samples of the placebo and test arm as well as between day 1 and day 15 (Fig. 4).

3.2.2 Change in serum insulin and CRP level

Serum insulin was analysed on day 15 at 0 h and 4 h of the post ingestion of the supplement. Placebo arm showed that the insulin concentration was increased significantly after 4 h (14.57 ± 4.02 µU/ml to 37.17 ± 6.18 µU/ml, $p = 0.0043$),

Table 3 AUC and C_{max} for plasma amino acid concentration of placebo (Whey protein + maltodextrin) and test (Whey protein + Pepzyme Pro) on day 1

Amino acids	Placebo				Test				*p* value		Increase (%)	
	AUC (µmol*h/L)		C_{max} (µmol/L)		AUC (µmol*h/L)		C_{max} (µmol/L)		AUC	C_{max}	AUC	C_{max}
	Mean	SD	Mean	SD	Mean	SD	Mean	SD				
Alanine	2710	846	823	272	2534	1193	814	473	0.647	0.951	−6.46	−1.05
Arginine	180	149	58	46	158	65	55	25	0.592	0.806	−12.61	−5.77
Asparagine	475	177	156	59	459	194	159	76	0.819	0.913	−3.30	1.74
Aspartic acid	3.9	2.0	1.2	0.6	4.1	2.3	1.3	0.8	0.735	0.744	6.90	7.02
Cystine	181	43	55	15	195	42	59	11	0.357	0.431	8.04	6.95
Glutamine	194	84	65	27	197	88	64	29	0.933	0.936	1.37	−1.29
Glutamic acid	3485	1214	1043	402	3467	1671	1090	632	0.974	0.811	−0.51	4.48
Glycine	1379	693	414	198	1503	794	459	264	0.652	0.598	8.98	11.00
Histidine	317	89	98	32	317	143	101	59	0.993	0.841	0.13	3.58
Isoleucine	625	280	239	113	586	255	234	112	0.690	0.894	−6.30	−2.31
Leucine	1023	551	366	194	1037	591	401	246	0.948	0.667	1.34	9.63
Lysine	711	287	270	136	751	314	271	123	0.721	0.985	5.57	0.33
Methionine	97	34	36	15	109	56	41	25	0.485	0.515	12.27	13.70
Phenylalanine	290	143	94	59	296	171	97	68	0.915	0.878	2.14	3.84
Proline	1322	422	408	157	1306	526	400	169	0.929	0.898	−1.19	−1.89
Serine	418	142	130	45	462	262	150	91	0.575	0.464	10.46	14.97
Threonine	664	243	213	76	739	345	245	125	0.502	0.404	11.15	15.02
Tryptophan	316	129	108	47	378	197	134	74	0.317	0.261	19.55	24.03
Tyrosine	287	152	95	46	322	174	110	64	0.567	0.466	11.99	15.90
Valine	1275	613	417	199	1449	684	482	260	0.469	0.445	13.65	15.69
Total	15,954				16,268						1.97	
Total BCAA	2923				3071						5.08	
Total EAA	5318				5661						6.44	

whereas test arm showed statistically insignificant increase after 4 h of the supplement consumption (22.68 ± 9.06 µU/ml to 35.09 ± 8.09 µU/ml, $p = 0.329$). There was no observed change in the CRP level between placebo and test as well as between 0 and 4 h (Fig. 5).

3.3 Change in the gut microbiome

Change in gut microbiome profiles between Day 1 (WP_Pre) and Day 15 (WP_Post) of supplementation was characterized and visualized for direct quantitative comparison of abundances, alpha and beta diversity measures, and differentially abundant species. After the whole metagenomic sequencing, the obtained composition of kingdoms is shown as stacked bar plots (Fig. 6A). Though, any significant difference was not observed in the composition; some shift in abundance of few groups was observed. Largely, bacterial and viral abundances showed upward trend by Day 15 of supplementation, from, 99.61% (WP_Pre) to 99.65% (WP_Post) ($p = 0.17$) and 0.12% (WP_Pre) to 0.17% (WP_Post) ($p = 0.21$) respectively. Inversely, archaeal and eukaryotic abundance showed downward trend by Day 15 of supplementation, from 0.04% (WP_Pre) to 0.02% (WP_Post) ($p = 0.25$) and 0.24% (WP_Pre) to 0.16% (WP_Post) ($p = 0.58$) respectively. This pattern continued at phylum level (Fig. 6B), with minor increase in abundance of *Bacteroidetes*, and decrease in abundance of *Firmicutes* and *Actinobacteria* post supplementation.

The increase in *Bacteroidetes* within WP_Post samples was in turn due to increase in abundance of species of *Phocaeicola* (*Phocaeicola plebeius* and *Phocaeicola coprocola*) and *Prevotella* (*Prevotella copri* and *Prevotella hominis*) (Fig. 6F). While decrease in *Fir`micutes* was largely due to reduction in relative abundance of *Streptococcus lutetiensis* and *Streptococcus equinus*, the reduction in *Actinobacteria* was due to reduced abundance of *Atopobium sp.* (Fig. 6F), and some *Bifidobacterium* species (like *Bifidobacterium longum* and *Bifidobacterium subtile*). Other than these, *Elusimicrobium_sp*_An273, belonging to the phylum of *Elusimicrobia* was found to be decreased in the WP_Post samples to a great extent (Fig. 6F). Lastly, crAssphages contributed largely to the change in viral abundance, with crAssphage_cr124_1 and

Table 4 AUC and C_{max} for plasma amino acid concentration of placebo (Whey protein + maltodextrin) and test (Whey protein + Pepzyme Pro) on day 15

Amino acids	Placebo				Test				*p* value		Increase (%)	
	AUC (µmol*h/L)		C_{max} (µmol/L)		AUC (µmol*h/L)		C_{max} (µmol/L)		AUC	C_{max}	AUC	C_{max}
	Mean	SD	Mean	SD	Mean	SD	Mean	SD				
Alanine	2622	1103	759	302	2577	647	935	430	0.893	0.206	−1.7	23.14
Arginine	245	129	82	42	299	184	95	57	0.355	0.488	22.26	15.68
Asparagine	481	257	149	75	580	362	178	106	0.395	0.401	20.58	19.19
Aspartic acid	4.1	1.9	1.3	0.7	4.1	2	1.3	0.5	> 0.999	0.887	0	−2.45
Cystine	147	42	47	16	132	55	42	16	0.416	0.35	−10.05	−11.81
Glutamine	208	93	70	29	212	113	66	32	0.927	0.781	1.67	−4.48
Glutamic acid	3549	1826	1015	511	3714	1635	1086	467	0.796	0.693	4.65	7.02
Glycine	1282	538	377	176	1238	355	373	148	0.791	0.952	−3.48	−0.95
Histidine	630	690	465	668	626	547	343	501	0.983	0.576	−0.76	−26.26
Isoleucine	606	279	209	79	733	357	266	146	0.288	0.192	20.92	27.55
Leucine	880	514	309	193	937	361	326	131	0.727	0.779	6.49	5.54
Lysine	851	257	287	66	987	369	327	121	0.25	0.273	16.04	13.85
Methionine	107	35	35	10	126	45	42	16	0.221	0.193	17.09	18.63
Phenylalanine	236	78	68	22	249	79	71	22	0.665	0.654	5.31	5.39
Proline	1533	700	450	198	1545	678	449	213	0.964	0.987	0.75	−0.27
Serine	378	132	110	38	430	143	127	45	0.307	0.275	13.86	15.19
Threonine	629	235	188	64	746	302	218	82	0.248	0.263	18.56	16.23
Tryptophan	306	97	94	28	343	87	105	28	0.278	0.29	12.14	11.67
Tyrosine	291	123	91	41	283	84	86	27	0.85	0.714	−2.52	−5.22
Valine	1145	581	374	222	1008	309	300	87	0.427	0.238	−11.97	−19.87
Total	16,129				16,767						3.95	
Total BCAA	2630				2677						1.78	
Total EAA	5390				5753						6.74	

Table 5 T_{max} (h) for plasma amino acid concentration of placebo (Whey protein + maltodextrin) and test (Whey protein + Pepzyme Pro) on day 1

Amino acids	Placebo		Test		*p* value
	Mean	SD	Mean	SD	
Alanine	1.6	0.8	1.4	1.0	0.689
Arginine	1.6	1.0	1.4	0.9	0.507
Asparagine	1.5	0.6	1.6	1.1	0.626
Aspartic acid	1.0	1.1	1.4	1.1	0.365
Cystine	1.5	1.4	1.4	1.1	0.889
Glutamine	1.3	1.2	1.0	1.3	0.619
Glutamic acid	1.9	1.1	1.8	1.3	0.817
Glycine	0.8	0.8	1.1	1.1	0.347
Histidine	1.3	0.8	1.7	1.1	0.317
Isoleucine	1.4	0.8	1.4	0.7	> 0.999
Leucine	1.4	0.7	1.3	0.8	0.728
Lysine	1.4	0.8	1.3	0.6	0.897
Methionine	1.4	0.8	1.3	0.9	0.583
Phenylalanine	1.3	0.8	1.6	1.2	0.481
Proline	1.5	0.7	1.7	1.1	0.560
Serine	1.4	0.8	2.2	0.9	0.022
Threonine	1.6	0.8	1.9	1.0	0.430
Tryptophan	1.7	0.6	1.6	0.9	0.734
Tyrosine	1.4	0.8	1.4	0.9	0.915
Valine	1.5	1.0	1.7	0.9	0.635

Table 6 T_{max} (h) for plasma amino acid concentration of placebo (Whey protein + maltodextrin) and test (Whey protein + Pepzyme Pro) on day 15

Amino acids	Placebo		Test		*p* value
	Mean	SD	Mean	SD	
Alanine	1.9	1.3	1.7	1.2	0.669
Arginine	1.7	1.1	1.5	1.2	0.640
Asparagine*	1.6	1.1	1.0	0.4	0.065
Aspartic acid	1.8	1.3	2.0	1.5	0.657
Cystine	2.1	1.4	1.4	1.3	0.181
Glutamine	1.6	1.4	1.2	1.6	0.464
Glutamic acid	1.4	0.8	1.2	0.7	0.546
Glycine**	1.1	0.7	0.6	0.3	0.016
Histidine	1.7	1.2	1.7	1.2	0.940
Isoleucine	1.3	0.6	1.1	0.5	0.316
Leucine*	1.5	0.9	1.0	0.4	0.074
Lysine	1.1	0.5	1.0	0.5	0.466
Methionine**	1.6	1.1	0.9	0.2	0.018
Phenylalanine	1.4	0.7	1.0	0.9	0.244
Proline	2.0	1.2	1.5	0.8	0.221
Serine	1.4	0.5	1.3	1.0	0.912
Threonine	1.9	0.8	2.0	0.9	0.682
Tryptophan	1.6	0.7	1.4	0.5	0.242
Tyrosine	1.8	0.8	1.4	0.7	0.146
Valine**	1.9	0.7	1.2	0.9	0.029

** and * represent significant difference between placebo and test arm at $p \leq 0.05$ and $p \leq 0.1$ respectively

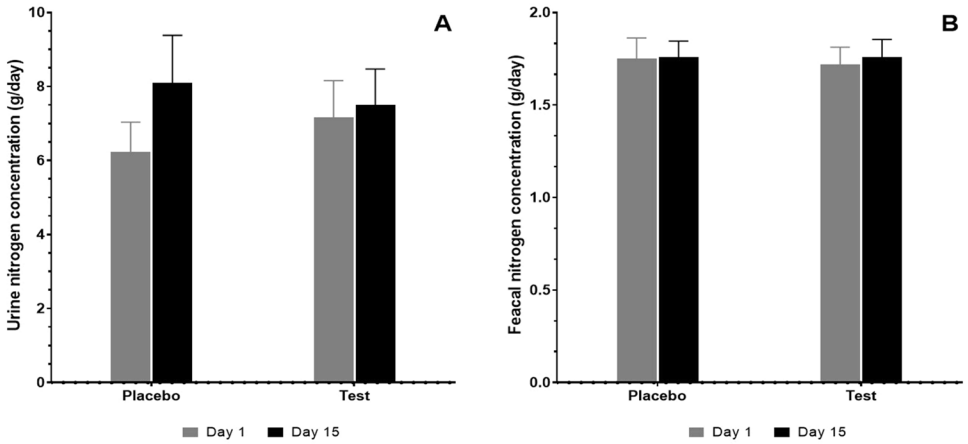

Fig. 4 Total nitrogen content (g/day) after consumption of whey protein + maltodextrin (placebo) and whey protein + Pepzyme Pro (test) present in urine (**A**) and faecal (**B**) samples. Values represented as mean ± standard error

crAssphage_cr7_1 increased, while crAssphage_cr85_1, crAssphage_cr130_1, and crAssphage_cr116_1 decreased in the WP_Post samples, overall leaning towards higher viral abundance post supplementation (Fig. 6F).

Both alpha and beta diversity measures further confirmed these minor shifts, with no significant difference in diversity measures between WP_Pre (Day 1) and WP_Post (Day 15). Chao1 (Fig. 6C) indicated a slight increase in species richness after supplementation with the diversity becoming more similar across all samples (lower deviation across samples). On the other hand, Shannon diversity measure (Fig. 6D) indicated a slight decrease in diversity after supplementation with almost similar evenness as compared to day 1 samples. The beta diversity measure by Bray–Curtis distance didn't establish any significant difference between Day 1 and Day 15 of supplementation. However, it displayed diverging clustering between the groups, represented by the ellipses in Fig. 6E.

Although there were no statistically significant changes in microbiome profile at the higher taxonomic levels, several species specific changes were observed that were statistically and functionally significant. Based on the consensus approach employed with five different DA tools, we could establish a total of 8 species to be significantly differentially

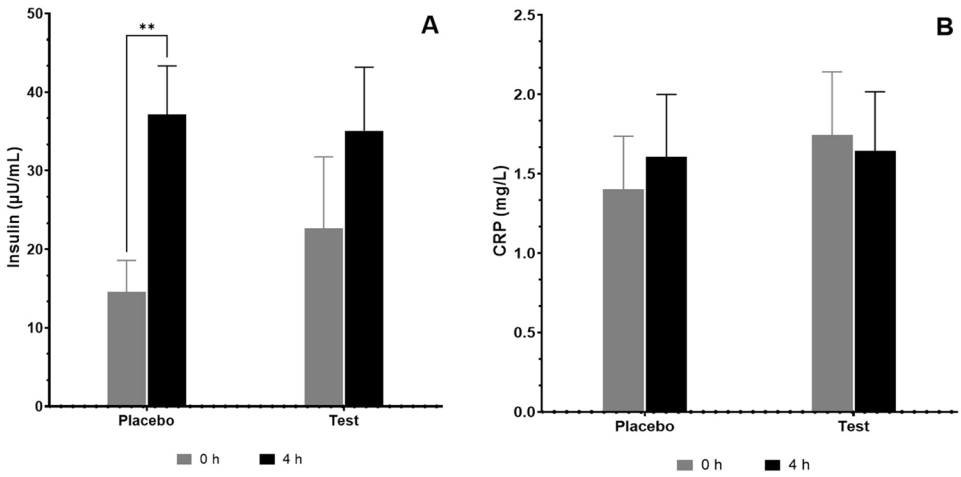

Fig. 5 Insulin (**A**) and CRP (**B**) levels in placebo (Whey protein + maltodextrin) and test (whey protein + Pepzyme Pro) on day 15. Values represented as mean ± standard error. ** represent significant difference between 0 and 4 h samples at $p \leq 0.05$

abundant ($p < 0.01$) between Day 1 and Day 15 samples. The comparative abundance plots of *Bacteroides acidifaciens*, *Bacteroides finegoldii*, *Bacteroides ovatus*, *Bacteroides sp.* 224, *Clostridium sp.* AF34 10BH, *Clostridium sp.* AM42 4, *Clostridium sp.* OF13 4, *Phocaeicola coprophilus* are illustrated in Fig. 6G (1–8).

4 Discussion

The present placebo controlled, crossover pilot clinical study was designed to evaluate the effect of a blend of enzymes and probiotics supplement (Pepzyme Pro) added to the whey protein on the amino acid absorption. Further, study evaluated the effect on the urine and fecal nitrogen level, insulin, CRP, and the gut microbiota. 15 healthy subjects were ingested with 30 g of whey protein along with the Pepzyme Pro (30 mg) or placebo (30 mg) for 15 days. On both day 1 and day 15, the test arm showed upward trend in the rate of absorption of amino acids compared to that of the placebo arm. Specifically, the absorption rate of isoleucine, leucine, and methionine were significantly higher in the test arm than the placebo arm on day 15. Besides, consumption of whey protein along with enzymes and probiotics blend for 15 consecutive days demonstrated the likelihood of augmenting the total absorption of essential and branched chain amino acids within 30 min of protein consumption (isoleucine 100%, leucine 161% and valine 51% on day 15). Test arm showed tendency to increase AUC and C_{max} as compared to the placebo arm on both the days of analysis. Enzymes and probiotics blend was effective in decreasing the T_{max} of glycine ($p = 0.016$), methionine ($p = 0.018$), and valine ($p = 0.029$) as well as showed propensity to reduce T_{max} of other amino acids on day 15. Overall, result demonstrated that the effect of enzymes on amino acid absorption was limited as observed on day 1, whereas consumption of supplement for 15 days illustrated that the enzyme and probiotics worked in harmony to increase the bioavailability of amino acids and hence can be a useful nutritional strategy to enhance the digestion of protein that would be beneficial to the athletes as well as lifestyle users.

The positive effect of external proteases on the protein absorption has been documented in our earlier in-vitro study [20]. Furthermore, *Bacilllus clausii* 088AE present in the supplement had individually shown to aid in protein digestion by inducing hydrolysis of dietary proteins [21]. Supplemented proteases assist and/or work in synchrony with the body proteases to break down the protein to generate lower molecular weight peptides, those were hypothesized to increase the gastric emptying leading to better absorption in the intestine [20]. The results of our clinical study are in good agreement with our previous in-vitro study in terms of faster gastric emptying and hence faster rate of absorption of the amino acids through the intestine. Probiotics regulate the intestinal microflora and thus influence the gut microbiota related to proteolysis. They are known to induce digestive proteases in the host and also can secrets proteases. Furthermore, they can improve the absorption ability of the epithelium and enhance the transport of the amino acids. They can reduce harmful protein fermentation in the colon and hence decrease the toxicity of the metabolites [22].

Supplementation of amino acid mixture, specifically branched chain amino acids, showed benefits in muscle function, fatigue, antioxidant capacity, and recovery in exercising athletes [23, 24]. This study provides inkling of increasing total amino acid absorption after supplementation of Pepzyme Pro with the whey protein. This will stimulate muscle protein synthesis without a need to increase the consumption of whey protein. In a clinical trial on 33 healthy men,

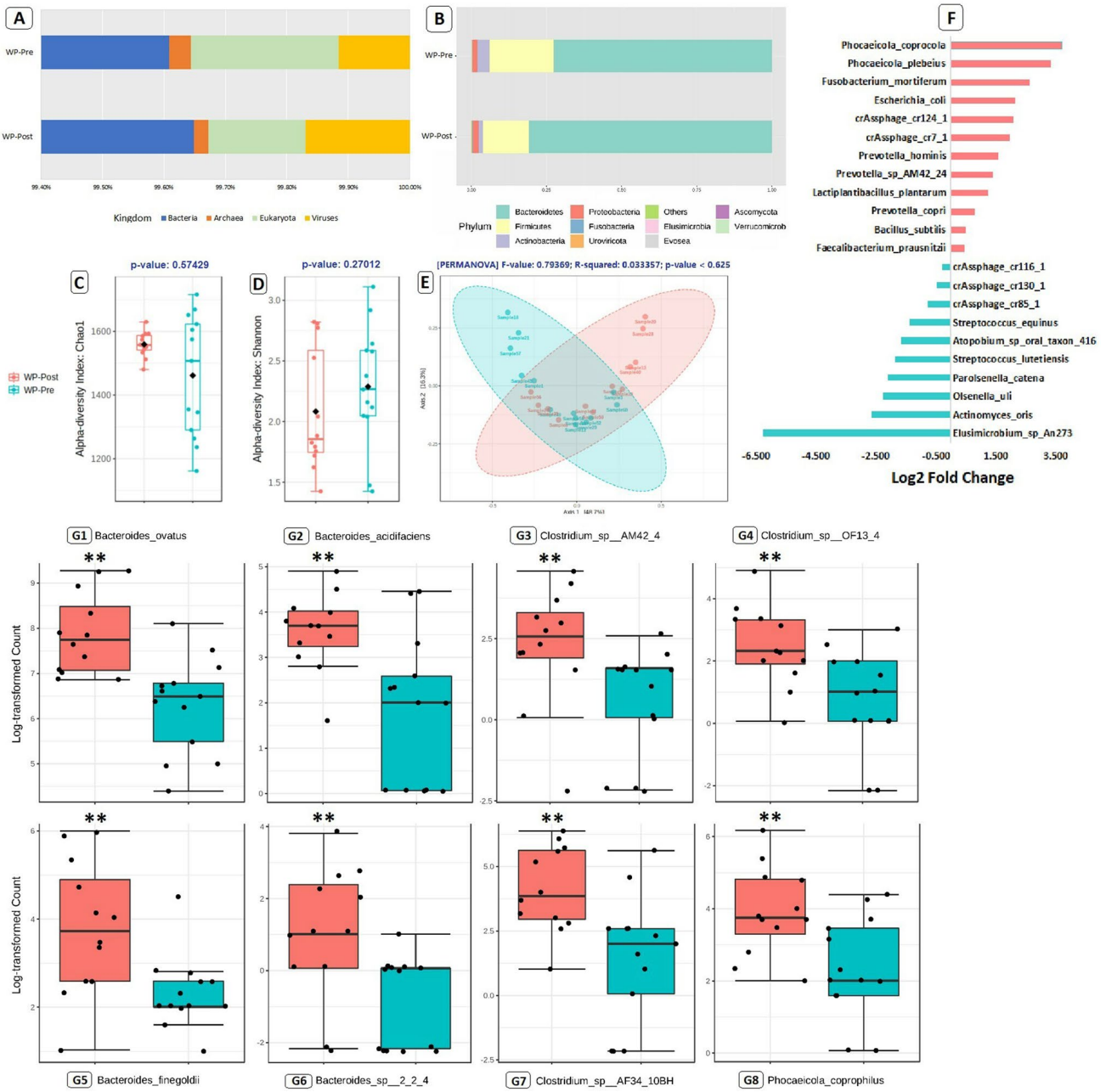

Fig. 6 **A** Quantitative comparison of abundances Kingdom level; **B** Quantitative comparison of abundances Phylum level; **C** Alpha Diversity—Chao1; **D** Alpha Diversity—Shannon Index; **E** Beta Diversity—Bray–Curtis distance, PERMANOVA; **F** Log2 Fold Change, of some of most differentially abundant species; G1-8: Differential abundance of eight species that were significantly ($p < 0.01$) differentially abundant

higher whey protein consumption (35 g) was found to be associated with the greater amino acid absorption and subsequent stimulation of de novo muscle protein synthesis compared to the ingestion of 10 or 20 g whey protein [25]. Besides muscle building and recovery function, amino acids are important for other health benefits such as glutamine has beneficial immune-based effects [26], proline plays important role in wound healing, antioxidation reactions, and immune responses [27], and serine has central role in cellular proliferation [28]. No difference was observed in the CRP level of the test arm and placebo arm suggesting the supplement is safe and has no negative effect on the immune functions of the body. Insulin concentration was increased significantly at 4 h in the placebo arm but not in the test arm after consumption of whey protein. Dietary proteins have an insulinotropic effect and thus promote insulin secretion [29]. However, insulin secretion might have been induced earlier in the test arm due to the faster absorption of amino acids as can be seen by lower T_{max}. This has not been captured in the data as the study did not include insulin concentration

kinetics. No significant difference was observed in the urine nitrogen and fecal nitrogen between the placebo and test arm demonstrating that the Pepzyme Pro did not impact the natural metabolic processes of the body and hence can be considered as a safe supplement.

The consumption of whey protein for a long time has been associated with the likelihood of negative impact on the gut microbiota [30]. Here, we have studied the impact of 15 days consumption of whey protein along with Pepzyme Pro on the gut microbiota. Microbiota profiling data showed a downtrend in Archaea abundance after consumption of whey protein and Pepzyme Pro. This is interesting as previous study with only whey protein supplementation showed an increase in Archaea diversity [31]. Putative role of the Archaea in intestinal diseases and its possible role as modulators of trimethylamine N-oxide (TMAO) concentration suggests that the supplementation might have a beneficial role in maintaining intestinal health. However, this would need further experimentation especially with respect to the role of the Archaea. Increased *Bacteroidetes* phylum was observed after 15 days of supplementation. It is known that some species belonging to this phylum have proteolytic activity [32] and hence can be speculated to be increased on the consumption of protein. Interestingly, several species of the phylum *Bacteroidetes*, such as *Bacteroides acidifaciens*, *Bacteroides finegoldii*, and *Bacteroides ovatus*, all of which are known to be beneficial commensals of the human gut microbiome, were found to have significantly increased abundance after supplementation. Further, two of the most numerically differential species *Phocaeicola dorei* and *Phocaeicola coprocola* (Fig. 6E), (previously known as *Bacteroides dorei* and *Bacteroides coprocola*) were increased in abundance post supplementation. *Bacteroides acidifaciens* is an acetate (a short chain fatty acid—SCFA) producing species [33], a potential immune system modulator, inducer of IgA [34] and can increase IL-6 and IL-10 production [35]. Some strains of *Bacteroides ovatus* are already considered as a potential next-generation probiotics due to its preventive effects on lipopolysaccharides-associated inflammation and intestinal microbiota disorders [36, 37]. It is also a versatile gut microbe in terms of nutrient utilization and management of environmental stressors. It has been demonstrated to produce a variety of SCFAs and synthesizes the neuro-active compounds gamma-aminobutyric acid (GABA) [38, 39]. The overall shift in microbial composition and abundance, with significant change in abundance of species of genus *Bacteroides* and phylum *Bacteroidetes* can largely be beneficial to promote healthy gut microbiota. These results illustrated that the consumption of Pepzyme Pro along with the protein would improve the gut microbiota even after consumption of a high protein diet.

Enzymes and probiotics taken as a supplement have also been reported for other health aspects. Protease supplementation after eccentric exercise is important to attenuate the muscle strength losses by regulating leukocyte activity and inflammation [40]. Bromelain supplementation had reduced fatigue in cyclists [41]. Further, protease supplementation facilitated muscle healing allowing for faster restoration of contractile function after intense exercise [42]. Probiotic supplementation was proven effective in decreasing zonulin in feces, a marker indicating enhanced gut permeability and beneficially affecting TNF-α and exercise induced protein oxidation [43]. This pilot clinical trial demonstrated a possibility of the improved amino acid absorption and balanced gut microbiome after consumption of Pepzyme Pro along with the whey protein. However, limited subject size impedes statistical analysis. Every individual varies in the metabolic rates and the digestive processes hence the large subjects size might help to prove the results statistically significant. Insulin and CRP analysis were at the fixed point hence all point analysis would be important to draw the conclusion. Analysis of short chain fatty acids would also be important to shed more light on the effect on the gut microbiota.

5 Conclusions

This double blind, crossover, and randomized pilot clinical study adds to the body of knowledge describing the effect of enzymes and probiotics blend added in the whey protein on the amino acid absorption and gut microbiota changes. Consumption of whey protein with enzymes and probiotics blend for 15 days could show upward trend in rate of absorption as well as total absorption of the amino acids that has been associated with the plethora of health benefits. Absorption of the essential amino acids and branched chain amino acids within 30 min of post ingestion of protein was found to be in upward trend due to Pepzyme Pro. Though, a full scale clinical study on large sample size is warranted to confirm the results statistically, this study demonstrated that Pepzyme Pro could potentially improve the amino acid absorption and gut microbiota by promoting the rebalance of the intestinal ecosystem.

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Author contributions Conceptualization, methodology: AR, and SJ; Data curation, formal analysis: TG, SJ, DD, and GK; Visualization: AR, SJ, GK, TG; Writing—original draft: SJ, TG, GK.; Writing—review and editing: AR, DD.

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Data availability The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1023559.

Declarations

Informed consent Informed written consent was obtained from all participants. No vulnerable subject participated in the study.

Competing interests AR, SJ, and TG are paid employees of Advanced Enzyme Technologies, which sponsored the study and has a corporate affiliation with Specialty Enzymes and Probiotics. Specialty Enzymes and Probiotics had no role in study design and actual conduct of the study. DD and GK have no conflict of interest in the study.

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