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Original Research

Protein substitutes used in the treatment of phenylketonuria distinctly modulate gut nutrient absorption and bacterial growth: An *in vitro* study

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ABSTRACT

Low phenylalanine (Phe) protein substitutes (PS), either L-amino acids (L-AAs) or casein glycomacropeptide with added amino acids (CGMP-AAs) are the mainstay of phenylketonuria (PKU) treatment. Given the central role of intestinal nutrient absorption and gut microbiota in metabolic regulation, this study investigated how different PS affect nutrient absorption and bacterial growth. It was hypothesized that L-AAs and CGMP-AAs would exert distinct effects. Caco-2 cells, an *in vitro* model of enterocytes, were used to assess the acute effects of PS and whey protein (WP) on glucose, amino acids, and fatty acids analogues up-

Abbreviations: 2-NBDG, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino; AT, annealing temperature; AUC, area under the curve; BCFAs, branched-chain fatty acids; BSA, bovine serum albumin; CD36, cluster of differentiation 36; CFU, colony-forming units; CGMP, casein glycomacropeptide; CGMP-AAs, casein glycomacropeptide with amino acids; DMSO, dimethyl sulfoxide; DWP, digested whey protein; EDTA, ethylenediaminetetraacetic acid; F, forward; FAT, fatty acid translocase; FATP4, fatty acid transport protein 4; FBS, foetal bovine serum; FSA, fluorescein sulfonic acid; GLUT 2, glucose transporter 2, HPLC, high-performance liquid chromatography; HPRT, hypoxanthine guanine phosphoribosyltransferase; KRP, Krebs-Ringer phosphate; L-AAs, phe-free L-amino acids supplement; LAT1, L-type amino acid transporter 1; LNAAs, large neutral amino acids; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD_{600}, Optical Density at 600 nm; PAH, phenylalanine hydroxylase; PBS, phosphate-buffered saline; PEPT1, peptide transporter 1; Phe, phenylalanine; PKU, phenylketonuria; PS, protein substitute; RT-qPCR, real-time quantitative reverse transcription polymerase chain reaction; R, reverse; R_{max}, maximum growth rate; SCFAs, short-chain fatty acids; SEM, standard error of the mean; SGLT1, sodium/glucose cotransporter 1; WP, whey protein.

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take, as well as the longer-term effects on nutrient transporter gene expression. The growth and metabolite production of *Escherichia coli*, *Lactobacillus paracasei*, and *Shigella flexneri* were also assessed. L-AAs reduced glucose absorption and downregulated glucose transporter 2 (GLUT2). Although L-AAs did not affect amino acids or fatty acids absorption, they downregulated L-type amino acid transporter 1 (LAT1) and peptide transporter 1 (PEPT1). CGMP-AAs had no effect on glucose or fatty acid absorption but impaired amino acid absorption and upregulated LAT1. These findings demonstrate formulation-specific effects on nutrient absorption and transporter gene expression. All PS increased bacterial abundance, with L-AAs producing the most pronounced effect relative to CGMP-AAs. L-AAs and WP accelerated the growth rate of all bacterial species, whereas CGMP-AAs selectively promoted the growth of *L. paracasei*, indicating a potential prebiotic-like effect. In conclusion, PS distinctly modulate intestinal nutrient transport and bacterial growth, underscoring the importance of considering their specific metabolic impact in PKU and the need for further translational research.

1. Introduction

Phenylketonuria (PKU; OMIM 261600) is an autosomal recessive inborn error of phenylalanine (Phe) metabolism mostly caused by variants in the gene encoding phenylalanine hydroxylase (PAH) [1]. PAH deficiency leads to accumulation of Phe in the blood and brain, which, if left untreated, results in neurocognitive and behavioural impairments [2,3].

Primary treatment is a lifelong low-Phe diet, supported by low Phe protein substitutes (PS) as the main source of protein equivalent [4]. PS are typically administered 3 to 4 times per day and are based on either Phe-free L-amino acids supplements (L-AAs), or low Phe casein glycomacropeptide supplemented with amino acids (CGMP-AAs) [3,5,6]. Casein glycomacropeptide (CGMP) is a bioactive peptide derived from cheese whey protein (WP) that is naturally low in Phe. However, as it lacks a complete amino acid profile, CGMP-based PS are fortified with essential amino acids and tyrosine [4,7].

In recent years, some studies (including both early- and late-diagnosed patients with PKU) have reported an increased prevalence of comorbidities, including diabetes mellitus, dyslipidaemia, obesity, and cardiovascular diseases [8–11]. However, it remains unclear if these metabolic comorbidities occur as a direct consequence of the disease itself, are associated with the Phe-restricted diet, or are an effect of the timing of diagnosis [12].

Intestinal absorption of key nutrients such as glucose, amino acids, and fatty acids, is essential for maintaining metabolic homeostasis and cellular function [13]. In enterocytes, specific transporters mediate the uptake of nutrients from the intestinal lumen and their subsequent release into circulation [13–15]. Increased intestinal absorption of glucose and fatty acids can result in an elevation of their plasma concentrations, which, when chronically sustained, could potentially contribute to hyperglycemia [16] and dyslipidemia, respectively [17]. Over time, these metabolic alterations may predispose to insulin resistance, type 2 diabetes mellitus, and cardiovascular diseases [18,19]. Investigating if PS modulates the intestinal nutrient absorption is therefore critical to unravel the mechanisms underlying the increased risk of metabolic comorbidities observed in patients with PKU. Moreover, given that these patients chronically consume PS mainly composed of free amino acids, it is crucial to determine if such intake alters intestinal amino acid absorption.

The gut microbiota is a complex population of microorganisms that inhabit the gastrointestinal tract [20]. Its composition is strongly shaped by diet, including protein sources, leading to the enrichment or depletion of specific microbes and their derived metabolites [20–22]. These microbial metabolites, including short-chain fatty acids (SCFAs) and branched-chain fatty acids (BCFAs), affect host physiological functions, such as energy homeostasis, glucose and lipid metabolism, and inflammation. Therefore, alterations in gut bacterial composition and the repertoire of metabolites produced have been associated with the development of several diseases, including obesity, type 2 diabetes and other metabolic disorders [23]. Taking this into consideration, the ability of PS to beneficially or detrimentally influence gut microbiota composition and its metabolites may impact gut health and gastrointestinal symptoms in PKU [24].

Considering the central role of intestinal nutrient absorption and gut microbiota in metabolic regulation, this study aimed to investigate the impact of L-AAs, CGMP-AAs, and WP on nutrient absorption using an *in vitro* model of enterocytes. In parallel, the influence of these protein sources on the growth and metabolite production of *bona fide* gut bacterial species, *Escherichia coli* (commensal), *Lactobacillus paracasei* (probiotic), and *Shigella flexneri* (pathogenic), was also evaluated. It was hypothesised that L-AAs and CGMP-AAs would exert distinct effects on both nutrient absorption and bacterial growth.

2. Materials and methods

2.1. *Protein substitutes preparation*

The powdered PS evaluated in this study were L-AAs (Milupa PKU 3 Advanced, Nutricia, Cuijk, Netherlands) and CGMP-AAs (Bettermilk, Cambrooke, MA, USA). Water served as the neg-

ative control, and WP (100% Real Whey Protein, Prozis, Esposende, Portugal) was used as a positive control. WP was selected because CGMP is derived from this intact protein [7] and thus serves as a reliable reference for comparison. The nutritional composition per 100g of each PS and WP is shown in Table 1. All PS and WP preparations were dissolved in water, and glucose was added to ensure equivalent carbohydrate concentrations across experimental conditions. Table 2 presents the final composition of each preparation adjusted to a total protein concentration of 10 mg/mL.

CGMP-AAs formulations typically contain 60%-70% of protein equivalent from CGMP, with the remaining protein provided by conventional L-amino acids [4,5]. Since enterocytes are capable of absorbing both free amino acids and small peptides, and express brush border peptidases that break down peptides before absorption [25], the components of CGMP-AAs formulations are expected to be directly absorbed, eliminating the need for prior enzymatic digestion. WP was submitted to an enzymatic digestion following the INFOGEST static *in vitro* digestion protocol [26] to mimic the gastrointestinal digestion process. The effectiveness of this digestion was confirmed using the Biuret-Gornall Protein Assay [27], which measures total protein concentration. This method showed a 39% reduction in total protein concentration in WP after enzymatic digestion (data not shown). The digested whey protein (DWP) obtained was subsequently tested in Caco-2 cells alongside other PS.

2.2. *Caco-2 cell culture*

The human epithelial cell line Caco-2 was obtained from the American Type Culture Collection (ATCC HTB-37 Rockville, MD, USA) and was used between passages 26 and 40. Caco-2 cells differentiate to form a polarized epithelial monolayer resembling small intestinal enterocytes. They exhibit key enterocyte features, including: 1) apical microvilli, 2) expression of tight junctions (e.g., claudins) and nutrient transporters (e.g., glucose, amino acid, and fatty acid transporters) and 3) activity of digestive enzymes (e.g., sucrose-isomaltase and peptidases), making them a well-established *in vitro* model for studying intestinal nutrient absorption [28]. Cell monolayers were cultured in Minimum Essential Medium at pH 7.4, supplemented with 15% foetal bovine serum (FBS), 25 mM HEPES, 25 mM $NaHCO_3$, 100 units/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (all from Sigma-Aldrich, Madrid, Spain). Cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2 - 95% air. For subculturing, cells were trypsinised using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich), for 1 minute at 37 °C, split at a 1:4 ratio, and seeded into plastic culture dishes (21.5 cm2; ∅ 60 mm; NEST Scientific Europe B.V, Hendrik-Ido-Ambacht, Netherlands).

Cell viability was assessed in 24-well plastic culture plates (1.93 cm2; ∅ 16.2 mm; Orange Scientific, Braine-l'Alleud, Belgium). For glucose, amino acids and fatty acids transport studies, and cell monolayer integrity assessment, cells were seeded in 12-well Transwell inserts (polycarbonate membrane, 0.4 µm pore size, ∅ 12 mm; Corning Costar, NY, USA). For RNA extraction, cells were seeded in 24-well plastic cell culture plates. Culture medium was changed every 2 - 3 days, and cells were split every 7 days. Cells were maintained 11-12 days in culture before experiments, after reaching 90 - 100% confluence, allowing spontaneous differentiation.

2.3. *Cell cytotoxicity assay*

The effect of PS and WP on Caco-2 cell cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [29]. The concentrations of the three protein sources were selected based on a previous study [30] and represent those typically found in the intestinal lumen [31].

Caco-2 cells were treated with water, L-AAs, cGMP-AA, DWP or WP for 2 h or 24 h in FBS-free culture medium. After the treatment period, the culture medium was removed, and cell monolayers were incubated at 37 °C for either 2 or 24 h in Hanks' buffer (137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM $MgCl_2$, 0.33 mM Na_2HPO_4, 0.44 mM NaH_2PO_4, 0.25 mM $CaCl_2$, 0.15 mM Tris.HCl, and 1.0 mM sodium butyrate [Sigma-Aldrich], pH=7.4) containing 5 mg/mL MTT (Thermo Fisher Scientific, Leiden, The Netherlands). After incubation, the MTT solution was carefully aspirated, and cells were solubilized in 200 µL of dimethyl sulfoxide (DMSO) (VWR, PA, USA) and subsequently diluted 4-fold. Absorbance was measured at 540 nm to quantify the formation of purple formazan crystals, generated by mitochondrial dehydrogenase-mediated MTT reduction. To correct for nonspecific MTT reduction, absorbance at 660 nm was subtracted from that of the 540 nm reading. Both absorbance values were recorded using a Spark microplate reader (Tecan, Männedorf, Switzerland). Results were expressed as a percentage of the control.

2.4. *Cell permeability assay*

The effect of PS and WP on the permeability of Caco-2 cell monolayers was assessed by measuring the apical-to-basolateral passage of fluorescein sulfonic acid (FSA). Since FSA crosses epithelial barriers exclusively via the paracellular route (i.e., through tight junctions), it is considered a sensitive and reliable biomarker of epithelial permeability and integrity [32].

Caco-2 cell monolayers were treated in the apical compartment of the Transwell insert with water, L-AAs, CGMP-AAs, DWP or WP, along with 100 µg/mL FSA (Thermo Fisher Scientific) in FBS-free culture medium, for 2 h. FSA fluorescence (excitation at 485 nm and emission at 530 nm) was measured on the basolateral compartment using a Spark microplate reader (Tecan). Results were expressed as a percentage of the control.

2.5. *Nutrient absorption assay*

The effect of PS and WP on nutrient absorption in Caco-2 cells was evaluated by measuring both the apical-to-basolateral transport over time (expressed as Area Under the Curve (AUC)), and the intracellular accumulation of fluorescent analogues of glucose, amino acids, and fatty acids. Following a 2-h incubation with water, L-AAs, CGMP-AAs, DWP or WP in FBS-free culture medium in the apical compartment of Transwell inserts, the culture medium was removed, and the cells were washed with phosphate-buffered saline (PBS) (VWR) before each specific nutrient absorption assay.

Table 1 – Nutritional composition of L-AAs, CGMP-AAs, and WP per 100 g.

	L-Aas	CGMP-AAs	WP
Calories, kcal	299	400	406
Protein Equivalent, g	70	38	91
Total Fat, g	0	11	3.4
Saturated Fat, g	0	4.6	0
Total Carbohydrate, g	4.7	37	2.9
Dietary Fiber, g	no information	0	no information
Added Sugars, g	no information	11	0
L-aspartate, mg	7800	1540	9800
L-carnitine, mg	140	0	no information
L-cysteine, mg	1900	19	2000
L-glutamate, mg	16600	3750	22000
L-glycine, mg	1900	2460	1200
L-histidine, mg	1900	860	1500
L-isoleucine, mg	4700	1970	7100
L-leucine, mg	7800	3940	12000
L-lysine, mg	5500	3340	8500
L-methionine, mg	1900	450	2000
L-phenylalanine, mg	-	38	2700
L-proline, mg	7500	2180	4900
L–serine, mg	4100	1220	4100
L-taurine, mg	no information	0	no information
L-threonine, mg	3700	3080	6000
L-tryptophan, mg	1400	950	1200
L-tyrosine, mg	5600	2920	2300
L-valine, mg	5500	1690	6600
L-alanine, mg	3400	1160	4400
L-arginine, mg	2700	3820	1900
Vitamin A, mcg RAE	575	710	no information
Vitamin C, mg	65	81	no information
Vitamin D, mcg	6.0	35	no information
Vitamin E, mg α-TE	9.5	12	no information
Vitamin K1, mcg	62	40	no information
Vitamin K2 (MK-7), mcg	no information	40	no information
Thiamine (B1), mg	1.2	1.5	no information
Riboflavin (B2), mg	1.3	1.5	no information
Niacin, mg NE	15.1	28	no information
Vitamin B6, mg	1.3	1.6	no information
Folate, mcg DFE	no information	335	no information
Folic acid, mcg	215	200	no information
Vitamin B12, mcg	3.0	2.8	no information
Pantothenic Acid (B5), mg	5.3	5.6	no information
Biotin, mcg	52	24	no information
Choline, mg	630	375	no information
Calcium, mg	1375	1350	no information
Chromium, mcg	22	33	no information
Copper, mg	0.5	0.95	no information
Iodine, mcg	230	165	no information
Iron, mg	13.4	16	no information
Magnesium, mg	355	170	no information
Manganese, mg	1.3	2.3	no information
Molybdenum, mcg	32	46	no information
Phosphorus, mg	685	1090	no information
Selenium, mcg	45	45	no information
Zinc, mg	15.5	7.5	no information
Sodium, mg	< 3.0	730	no information
Potassium, mg	1275	1230	no information
Chloride, mg	< 1.0	580	no information
Inositol, mg	310	44	no information
Salt, g	0	0	0.46

Abbreviations: α-TE - alpha-tocopherol equivalents; CGMP-AAs - casein glycomacropeptide with amino acids; DFE - dietary folate equivalents; L-AAs - phenylalanine-free L-amino acid supplements; MK-7 - menaquinone-7; NE - niacin equivalents; RA - retinol activity equivalent; WP - whey protein.

Table 2 – Nutritional composition of L-AAs, CGMP-AAs, and WP adjusted to 10 mg protein/mL.

	L-Aas	CGMP-AAs	WP
Amount of original product, mg	14.30	26.30	11.00
Calories, kcal	0.08	0.11	0.08
Protein equivalent, mg	10.00	10.00	10.00
Total fat, mg	0.0	2.90	0.37
Total carbohydrate, mg	9.70	9.70	9.70
L-aspartate, mg	1.11	0.41	1.08
L-carnitine, mg	0.02	0.00	no information
L-cysteine, mg	0.27	0.01	0.22
L-glutamate, mg	2.37	0.99	2.42
L-glycine, mg	0.27	0.65	0.13
L-histidine, mg	0.27	0.23	0.16
L-isoleucine, mg	0.67	0.52	0.78
L-leucine, mg	1.11	1.04	1.32
L-lysine, mg	0.79	0.88	0.93
L-methionine, mg	0.27	0.12	0.22
L-phenylalanine, mg	-	0.01	0.30
L-proline, mg	1.07	0.57	0.54
L–serine, mg	0.59	0.32	0.45
L-taurine, mg	no information	0.00	no information
L-threonine, mg	0.53	0.81	0.66
L-tryptophan, mg	0.20	0.25	0.13
L-tyrosine, mg	0.80	0.77	0.25
L-valine, mg	0.79	0.44	0.73
L-alanine, mg	0.49	0.31	0.48
L-arginine, mg	0.39	1.01	0.21

Abbreviations: CGMP-AAs - casein glycomacropeptide with amino acids; L-AAs - phenylalanine-free L-amino acid supplements; WP - whey protein.
Protein sources were dissolved in water, and glucose was added to ensure equivalent carbohydrate concentrations across all experimental conditions. For Caco-2 cell experiments, these stock solutions were used directly, while for bacterial growth assays, they were further diluted in water to achieve a final protein concentration of 0.3 mg/mL.

For the glucose absorption assay, cells were glucose-deprived for 30 minutes in Krebs-Ringer phosphate (KRP) buffer (20 mM HEPES, 137 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2.2H_2O$, and 1.5 mM sodium pyruvate [Sigma-Aldrich], pH = 7.4) containing 0.5% bovine serum albumin (BSA) (Merck, Darmstadt, Germany). Subsequently, cells were incubated with 200 µM 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) Amino (2-NBDG) (AAT Bioquest, California, USA) in the apical compartment, a fluorescent glucose analogue commonly used to monitor glucose uptake in live cells [33]. For the amino acid absorption assay, cells were incubated with 200 µM FITC-labeled D-Alanine (Sigma-Aldrich) in the apical compartment, a fluorescent derivative of D-alanine obtained through the synthetic conjugation of 3-amino-D-alanine to a fluorophore [34]. D-alanine was selected as a model substrate due to its small size, dietary abundance, and transport via multiple amino acid carriers, making it a suitable marker for evaluating potential alterations in amino acids absorption [35].

In both glucose and amino acid absorption assays, fluorescence in the basolateral compartment was measured at 30, 60, 90, and 120 minutes, while intracellular fluorescence was quantified at 120 minutes following cell lysis with 0.1% Triton X-100 (Merck). The resulting cell lysates were centrifuged at 8000 rpm for 1 minute. 2-NBDG fluorescence was measured in the supernatants at 465 nm (excitation) and 540 nm (emission), and FITC-labeled D-Alanine fluorescence at 495 nm (excitation) and 515 nm (emission), using a Spark microplate reader (Tecan).

Fatty acid absorption was evaluated using BODIPY-C_{12} fatty acid (Thermo Fisher Scientific), a 12-carbon saturated fatty acid linked to a fluorophore resembling an 18-carbon long-chain fatty acid [36]. Cells were incubated with 10 µM BODIPY-C_{12} and 0.1% defatted BSA (NZY Tech, Lisbon, Portugal) in the apical compartment. Basolateral fluorescence was measured at 1, 2, 3, 4, 5, and 6 h, and intracellular fluorescence was measured at 6 h following cell lysis with 5% IGEPAL (Sigma-Aldrich) in water. BODIPY-C_{12} fluorescence in cell lysates was measured in supernatants at 559 nm (excitation) and 568 nm (emission), using a Spark microplate reader (Tecan).

Transport kinetics were expressed as concentration *versus* time, and AUCs were calculated to provide an integrated measure of total transported nutrients over time. All fluorescence values were normalized to total protein content. Protein quantification after glucose and amino acid absorption assays was performed using the Bradford assay (absorbance at 595 nm), while protein quantification following the fatty acid absorption assay was carried out using the EZQ™ Protein Quantification Kit (Thermo Fisher Scientific), according to the manufacturers' instructions.

Table 3 – Primer sequences and annealing temperatures used for RT-qPCR.

Gene name	Primer sequence (5' to 3')	Annealing temperature (°C)
FAT/CD36	GGTACAGATGCAGCCTCATTTC (F) GCAAAGGCCTTGGATGGAAG (R)	60
FATP4	CTACCTTACTGGTGATGTGCTG (F) GCGGCTGAGTGTGCCTTCCAC (R)	60
GLUT2	CAG GAC TAT ATT GTG GGC TAA (F) CTG ATG AAA AGT GCC AAG T (R)	59
HPRT	TTTATGTCCCCTGTTGACTGG (F) TGCTGACCTGCTGGATTACA (R)	59
LAT1	TCCAGATCGGGAAGGGTGAT (F) AGAGGCCGCTGTATAATGCC (R)	60
PEPT1	ATGTCCGGGAAAATCGGAGC (F) CACAGCATCGAAGATCGGGA (R)	60
SGLT1	TGG CAA TCA CTG CCC TTT A (F) TGC AAG GTG TCC GTG TAA AT (R)	59

Abbreviations: FAT/CD36 - fatty acid translocase/cluster of differentiation-36; FATP4 - fatty acid transport protein 4; (F) - forward; GLUT2 - glucose transporter 2; HPRT - hypoxanthine-guanine phosphoribosyltransferase; LAT1 - L-Type Amino Acid Transporter 1; PEPT1 - peptide transporter 1; (R) – reverse; SGLT1 - sodium-glucose cotransporter 1.

2.6. RNA extraction and RT-qPCR

The gene expression of key intestinal nutrient transporters - glucose transporter 2 (GLUT2), sodium-glucose cotransporter 1 (SGLT1), L-type amino acid transporter 1 (LAT1), peptide transporter 1 (PEPT1), fatty acid translocase (FAT)/cluster of differentiation 36 (CD36) and fatty acid transport protein 4 (FATP4) - was assessed in Caco-2 cells by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR).

Caco-2 cells were treated with water, L-AAs, CGMP-AAs, DWP or WP for 24 h to mimic chronic exposure. Total RNA was extracted using NZYol Isolation Reagent following the manufacturer's protocol, and RNA purity was assessed spectrophotometrically using a Nanodrop spectrophotometer (Thermo Fisher Scientific), with A260/A280 ratios ranging between 1.85 and 2.00. For cDNA synthesis, 1 µg of total RNA was reverse transcribed using the NZY First-Strand cDNA Synthesis Kit, following manufacturer's instructions. Each individual RT-qPCR was performed using: 0.2 µM of each primer and NZYSupreme qPCR Green Master Mix (NZY Tech) according to manufacturer instructions. Thermocycling conditions were the following: denaturation (95 °C for 10 minutes), amplification and quantification (95 °C for 10 seconds, annealing temperature [AT] for 10 seconds and 72 °C for 10 seconds) repeated 45 times and a melting curve program (95 °C for 15 seconds, [AT + 10]°C for 60 seconds and 95 °C with a heating rate of 0.1 °C/s and continuous fluorescence measurement). Primer pairs used for amplification were designed using the primer designing tool – NCBI-NIH and synthesised by Invitrogen (Thermo Fisher Scientific). Their sequences and corresponding annealing temperatures are listed in Table 3. RT-qPCR was carried out in duplicate for each gene using the QuantStudio™ 5 device (Thermo Fisher Scientific), and the standard deviation of Cq values between duplicates was ≤ 0.4. Gene expression was normalized for the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT), and relative gene expression was calculated using the $2^{-}\Delta\Delta$Ct method.

2.7. Bacterial culture conditions and growth assay

Escherichia coli MG1655, *Lactobacillus paracasei* (ATCC 334), and *Shigella flexneri* M90T strain were generously provided by the Pasteur Institute Micro-organism Collection. These model strains were selected to represent distinct groups of gut bacteria: *E. coli* as a commensal species [37], *L. paracasei* as a probiotic [38], and *S. flexneri* as a pathogenic species [39], providing insight into the potential differential effects of PS on gut microbial growth. Prior to experiments, *E. coli* and *S. flexneri* were cultured on Mueller-Hinton broth (Milipore, Massachusetts, USA), and *L. paracasei* on Lactobacilli MRS Broth (Difco, Grenoble, France) at 37 °C overnight (approximately 12 to 18 h). On the day of the experiment, broth was supplemented with water, L-AAs, CGMP-AAs, DWP or WP to a final protein concentration of 0.3 mg/mL. This concentration was selected since it falls within the range of physiological protein concentrations found in the intestinal lumen [31] and, at the same time, minimizes spectrophotometric interference when measuring bacterial density. Bacterial suspensions were then inoculated in broth to achieve a final density of 10^6 colony-forming units (CFU)/mL and incubated at 37 °C for 24 h. Optical density was measured continuously at 600 nm (OD_{600}) every 30 minutes using a SPECTROstar Nano microplate reader (BMG Labtech, Ortenberg, Germany). Based on the continuous observation of the OD_{600}, the AUC in the exponential phase, and the maximal growth rate (R_{max}) were calculated. All experiments were conducted independently, on three separate days, for each bacterial strain.

2.8. Bacterial metabolite profiling

To evaluate the impact of different PS on bacterial metabolic activity, the production of a panel of metabolites, including kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, kynurenic acid, xanthurenic acid, quinolinic acid, 5-hydroxytryptophan, 5-hydroxyindole acetic acid, indole acetic acid, indole-3-acetamide, indole-3-carboxaldehyde, imida-

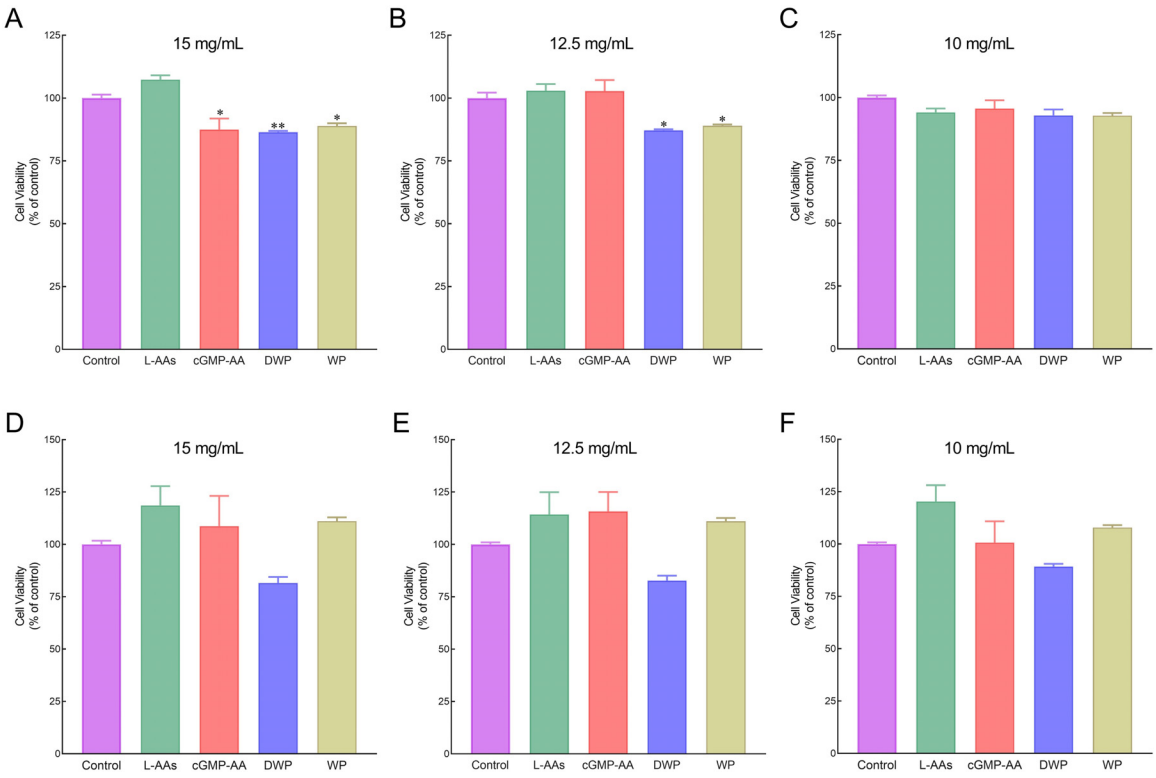

Fig. 1 – Exposing Caco-2 cells to protein substitutes (10 mg/mL) for 2 h (A, B, and C) and 24 h (D, E, and F) is not cytotoxic, as determined by the MTT assay. Data are presented as mean ± SEM (n = 6) *P < .05 vs. control and ** P < .001 vs. control (one-way ANOVA with post-hoc test). Abbreviations: L-AAs - phenylalanine-free L-amino acid supplements, CGMP-AAs - casein glycomacropeptide with amino acids, DWP - digested whey protein, WP - whey protein.

zole, indole propionic acid, indole, trimethylamine, as well as SCFAs (acetate, butyrate and propionate), and BCFAs (isobutyrate and isovalerate) was assessed in *E. coli*, *L. paracasei*, and *S. flexneri* culture supernatants after a 24-h incubation with water, L-AAs, CGMP-AAs, DWP or WP. Samples were derivatised and analysed by chromatographic separation and mass spectrometric detection using a Vanquish High-Performance Liquid Chromatography (HPLC) system coupled to an Orbitrap Exploris 120 mass spectrometer (Thermo Fisher Scientific).

2.9. *Statistical analysis*

Statistical analysis was performed using GraphPad Prism V8.0 (GraphPad Software, Inc., San Diego, CA, USA). Data are shown as mean ± standard error of the mean (SEM). The significance of differences between two groups was assessed using the Student's t-test, while comparisons involving three or more groups were evaluated using one-way or 2-way analysis of variance (ANOVA) followed by Dunnett's posthoc test. Statistical significance was accepted for $P < .05$.

3. Results

3.1. *Caco-2 cells cytotoxicity*

The cytotoxic potential of PS and WP on Caco-2 cells was assessed using the MTT assay (Fig. 1). A 2-h treatment with CGMP-AAs, DWP, and WP at 15 mg/mL significantly reduced cell viability compared to control conditions (Fig. 1A). Similarly, exposure to DWP and WP (but not to CGMP-AAs) at 12.5 mg/mL for 2 h also led to a significant decrease in cell viability compared to control (Fig. 1B). However, no significant changes were observed after a 24-h treatment at the above-mentioned concentrations (Figs. 1D and 1E). Treatment with L-AAs did not affect cell viability at any concentration or exposure time tested. Additionally, treating cells with PS and WP at 10 mg/mL did not affect cell viability at either 2 (Fig. 1C) or 24 h (Fig. 1F) treatment. Based on these findings, a concentration of 10 mg/mL was selected for subsequent experiments in Caco-2 cells.

3.2. *Caco-2 cells monolayer integrity*

To assess the impact of PS and WP on the integrity of the Caco-2 cell monolayer, the apical-to-basolateral passage of FSA was measured following a 2-h treatment. Compared to control conditions, PS and WP did not significantly alter FSA permeability, indicating that these formulations do not compromise monolayer integrity (Fig. 2).

3.3. *Glucose absorption in Caco-2 cells*

To assess the effect of the different protein treatments on glucose absorption in Caco-2 cells, both apical-to-basolateral transport (expressed as AUC) and intracellular accumulation

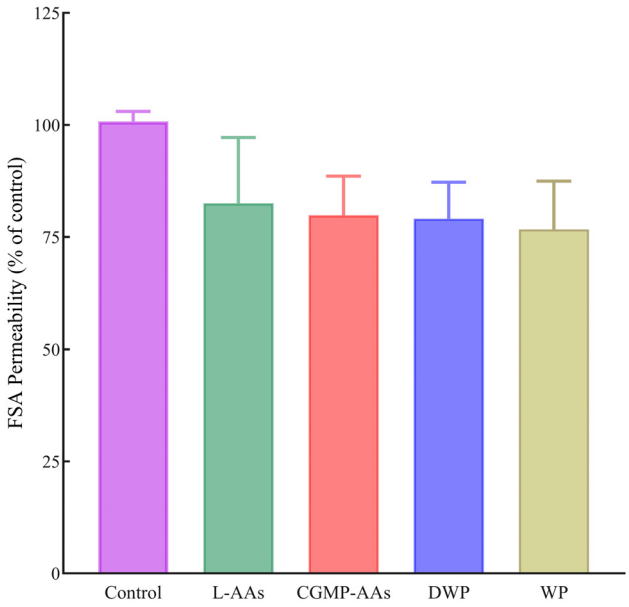

Fig. 2 – Acute exposure (2 h) of Caco-2 cells to different protein substitutes (10 mg/mL) does not compromise monolayer integrity, as determined by measuring the apical-to-basolateral passage of fluorescein sulfonic acid (FSA). Data are shown as mean ± SEM (n = 6). Abbreviations: L-AAs - phenylalanine-free L-amino acid supplements, CGMP-AAs - casein glycomacropeptide with amino acids, DWP - digested whey protein, WP - whey protein.

of 2-NBDG, a fluorescent glucose analogue, were measured. A 2-h exposure to WP and L-AAs reduced apical-to-basolateral 2-NBDG transport by 25%-30% compared to control conditions (Fig. 3A). In contrast, CGMP-AAs and DWP did not affect the apical-to-basolateral glucose transport. Moreover, none of the protein treatments altered intracellular 2-NBDG accumulation (Fig. 3B).

3.4. *Glucose transporters gene expression*

The gene expression of key intestinal glucose transporters, GLUT2 and SGLT1, was analyzed in Caco-2 cells by RT-qPCR. Chronic exposure (24 h) to WP induced a significant downregulation of GLUT2 and SGLT1 expression by 82% and 76%, respectively, compared to control conditions (Figs. 4A and B). In contrast, none of the other protein sources significantly altered the expression of these 2 transporters (Figs. 4A and B). A moderate, but nonsignificant ($p = .114$), decrease in GLUT2 expression (38%) was observed following chronic treatment with L-AAs compared to control conditions (Fig. 4A).

3.5. *Amino acid absorption in Caco-2 cells*

To evaluate the effect of PS and WP on amino acid absorption in Caco-2 cells, both apical-to-basolateral transport (expressed as AUC) and intracellular accumulation of FITC D-Alanine, a fluorescent derivative of D-alanine, were assessed. Acute exposure (2 h) to CGMP-AAs significantly reduced the apical-to-basolateral transport of FITC D-Alanine by 27% compared to control (Fig. 5A). Although intracellular FITC D-Alanine accumulation did not differ significantly between treatments, a general trend towards reduced intracellular accumulation was noted across all protein treatments compared to control conditions (Fig. 5B).

3.6. *Amino acid transporters gene expression*

The gene expression of the main intestinal amino acid and peptide transporters, LAT1 and PEPT1, was analysed in Caco-2 cells using RT-qPCR. Chronic exposure (24 h) to L-AAs, DWP, and WP resulted in a significant downregulation of LAT1 gene expression by 59%, 83% and 90%, respectively, compared to control conditions. In contrast, CGMP-AAs induced a significant 48% upregulation of LAT1 expression (Fig. 6A).

Regarding PEPT1, chronic exposure to L-AAs and WP significantly decreased its expression by 45% and 78%, respectively, while CGMP-AAs and DWP had no significant effect, compared to control conditions (Fig. 6B).

3.7. *Fatty acid absorption in Caco-2 cells*

To assess the effect of PS and WP on fatty acid absorption in Caco-2 cells, both apical-to-basolateral transport and intracellular accumulation of Bodipy-C_{12}, a fluorescently labelled long-chain fatty acid analogue, were measured. Treatment with different proteins for 2 h did not appear to affect fatty acid absorption in Caco-2 cells. No significant differences were observed in either apical-to-basolateral transport (Fig. 7A) or intracellular accumulation of Bodipy-C_{12} (Fig. 7B) compared to control conditions.

3.8. *Fatty acid transporters gene expression*

The gene expression of the main intestinal fatty acid transporters, FAT/CD36 and FATP4, was analysed in Caco-2 cells using RT-qPCR. Compared to control conditions, chronic exposure (24 h) to WP and DWP significantly downregulated FAT/CD36 expression by 63% (Fig. 8A), and FATP4 expression by 82% and 56% (Fig. 8B), respectively. In contrast, CGMP-AAs significantly upregulated FATP4 by 53%.

3.9. *Bacterial growth and abundance*

The impact of PS and WP on the growth of *E. coli*, *L. paracasei*, and *S. flexneri* was evaluated by calculating the AUC during the exponential bacterial growth phase, used as a proxy of bacterial final abundance or yield. All proteins (at 0.3 mg/mL) significantly increased the AUC of the three bacterial species compared to control conditions (Fig. 9). For all bacteria, this increase was more pronounced after exposure to L-AAs compared to CGMP-AAs (*E. coli*: 22.0% vs. 15.7%; *L. paracasei*: 31.8% vs. 17.2%; *S. flexneri*: 14.8% vs. 9.8%).

3.10. *Growth rate of bacteria*

To assess the impact of the different proteins on bacterial growth kinetics, the R_{max} was calculated during the exponential growth phase, an indicator of bacterial replication speed.

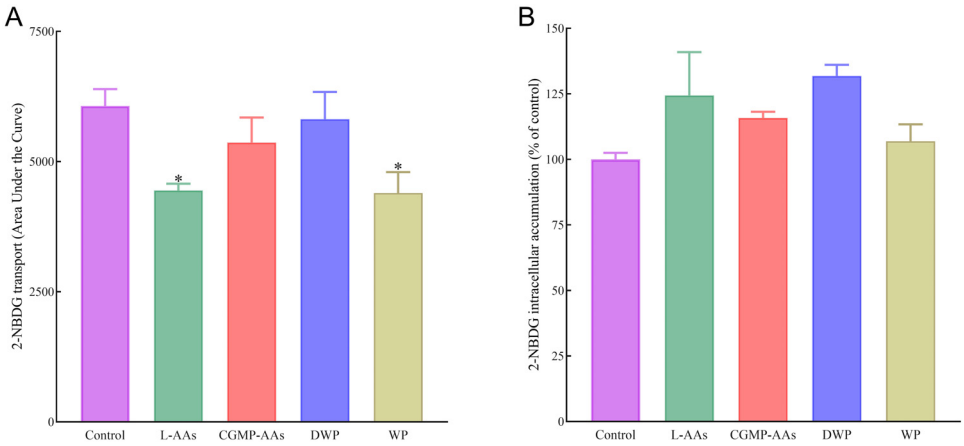

Fig. 3 – Acute exposure (2 h) of Caco-2 cells to L-AAs and WP (10 mg/mL) reduces apical-to-basolateral passage of 2-NBDG (A), without affecting intracellular accumulation of 2-NBDG (B). Data are shown as mean ± SEM (n = 4). * P < .05 vs. control (one-way ANOVA with post-hoc test). Abbreviations: L-AAs - phenylalanine-free L-amino acid supplements, CGMP-AAs - casein glycomacropeptide with amino acids, DWP - digested whey protein, WP - whey protein.

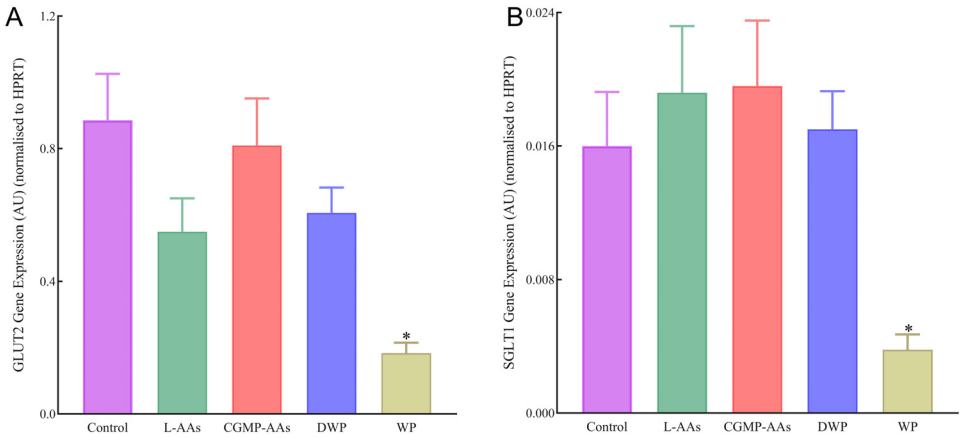

Fig. 4 – Chronic exposure (24 h) of Caco-2 cells to WP (10 mg/mL) downregulates glucose transporter 2 (GLUT2) (A) and sodium-glucose cotransporter 1 (SGLT1) (B) gene expression. Relative expression of these genes was evaluated by quantitative RT-qPCR. Results were normalized for the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Data are shown as mean ± SEM (n = 5-6). *P < .05 vs. control (one-way ANOVA with post-hoc test). Abbreviations: AU - arbitrary unit, L-AAs - phenylalanine-free L-amino acid supplements, CGMP-AAs - casein glycomacropeptide with amino acids, DWP - digested whey protein, WP - whey protein.

The growth rate was significantly increased by L-AAs, DWP, and WP for all bacteria compared to control (Fig. 10). Moreover, CGMP-AAs selectively increased the growth rate of *L. paracasei*.

3.11. *Bacterial metabolite production*

To evaluate the impact of PS and WP on bacterial metabolic activity, the production of a panel of metabolites was assessed by HPLC. Quantifiable concentrations were detected only for SCFAs, BCFAs, and indole, while the remaining metabolites were below the detection limits under the tested conditions (detailed results in Supplementary Material – Fig S1-S6). Overall, all three bacterial species produced SCFA, with no differences observed between treatments with protein sources after 24 h. The only exception was *E. coli*, whose acetate and propionate production increased after treatment with DWP and L-AAs, respectively. For BCFAs, *E. coli* treated with DWP and WP, and *S. flexneri* treated with WP, significantly increased isobutyrate and isovalerate production. Finally, both *E. coli* and *S. flexneri* produced indole after 24 h, with no differences between treatments, whereas *L. paracasei* did not produce detectable indole concentrations.

4. Discussion

Recent studies have highlighted an increasing prevalence of comorbidities in individuals with PKU, such as diabetes mellitus, dyslipidaemia, obesity, and cardiovascular diseases [8–11]. Taking this into account, and given the central role of intestinal nutrient absorption and gut microbiota in metabolic regulation, this study aimed to evaluate the effects of L–AAs,

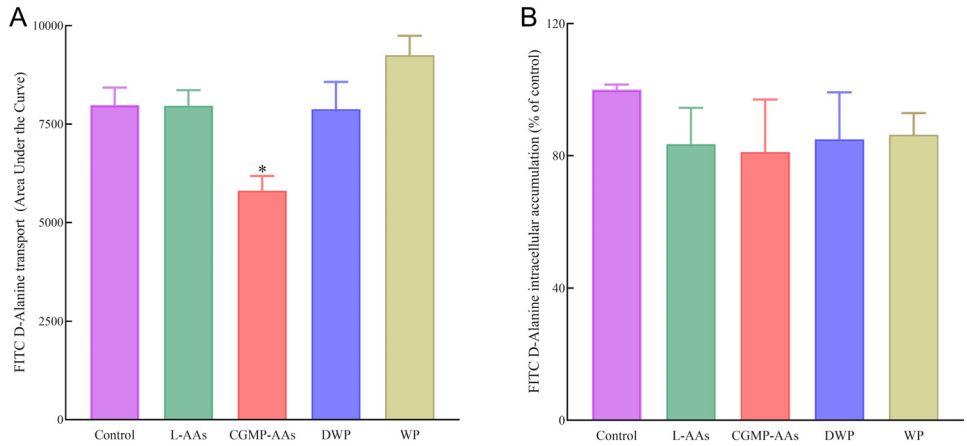

Fig. 5 – Acute exposure (2 h) of Caco-2 cells to CGMP-AAs (10 mg/mL) reduces apical-to-basolateral passage of FITC D-Alanine (A), without affecting intracellular accumulation of FITC D-Alanine (B). Data are shown as mean ± SEM (n = 4). *P < .05 vs. control (one-way ANOVA with post-hoc test). Abbreviations: L-AAs - phenylalanine-free L-amino acid supplements, CGMP-AAs - casein glycomacropeptide with amino acids, DWP - digested whey protein, WP - whey protein.

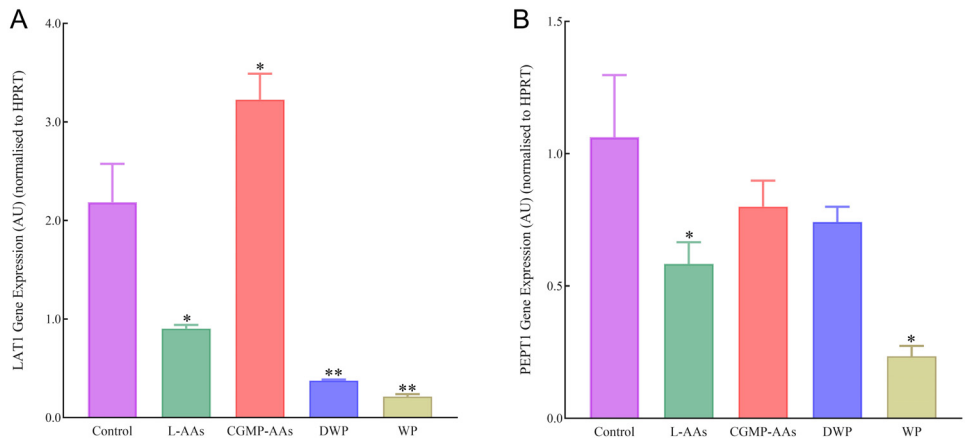

Fig. 6 – Chronic exposure (24 h) of Caco-2 cells to different protein substitutes (10 mg/mL) distinctly modulates L-Type Amino Acid Transporter 1 (LAT1) (A) and peptide transporter 1 (PEPT1) (B) gene expression. Relative expression of these genes was evaluated by quantitative RT-qPCR. Results were normalized for the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Data are shown as mean ± SEM (n = 5-6). *P < .05 vs. control and **P < .001 vs. control (one-way ANOVA with post-hoc test). Abbreviations: AU - arbitrary unit, L-AAs - phenylalanine-free L-amino acid supplements, CGMP-AAs - casein glycomacropeptide with amino acids, DWP - digested whey protein, WP - whey protein.

CGMP–AAs, and WP on nutrient absorption using an enterocyte cell model, as well as their impact on the *in vitro* growth of three representative gut bacterial species: *Escherichia coli*, *Lactobacillus paracasei*, and *Shigella flexneri*. The results confirmed the hypothesis that L–AAs and CGMP–AAs exert distinct effects on both nutrients' absorption and bacterial growth. While previous studies have assessed the impact of PS in Caco-2 cells, they primarily focused on oxidative and inflammatory stress [30,40], making the present study the first to investigate the effects of L-AAs and CGMP-AAs on nutrient absorption and transporter expression, as well as bacterial growth.

In this study, L-AAs exhibited a pronounced impact on both nutrient absorption and bacterial growth. In Caco-2 cells, L-AAs significantly reduced glucose absorption and moderately downregulated GLUT2 expression. This transporter is known to translocate from intracellular vesicles to the apical membrane of enterocytes in response to an increase in intestinal luminal glucose [41], a mechanism observed in individuals with obesity and linked to high caloric intake, insulin resistance, and elevated blood glucose concentrations [42]. Although the downregulation of GLUT2 by L-AAs was modest and not statistically significant, it may still contribute to reduce intestinal glucose absorption, potentially supporting a better glycaemic control. This is consistent with clinical observations in PKU showing that a higher protein intake, from both PS and natural protein, is protective against excessive weight gain during a 10-year period [43].

Furthermore, despite not altering amino acids and fatty acids absorption, L-AAs significantly downregulated LAT1 and

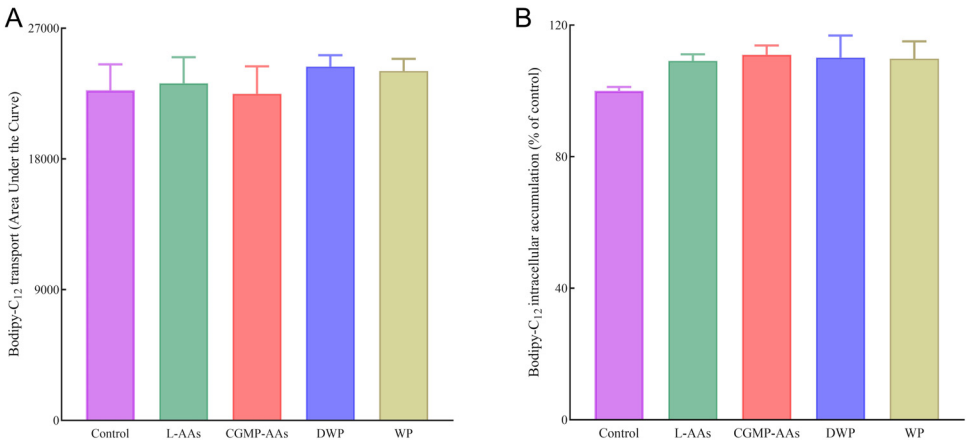

Fig. 7 – Acute exposure (2 h) of Caco-2 cells to different protein substitutes (10 mg/mL) does not change apical-to-basolateral passage (A) or intracellular accumulation (B) of Bodipy-C12. Data are shown as mean ± SEM (n = 4). Abbreviations: L-AAs - phenylalanine-free L-amino acid supplements, CGMP-AAs - casein glycomacropeptide with amino acids, DWP - digested whey protein, WP - whey protein.

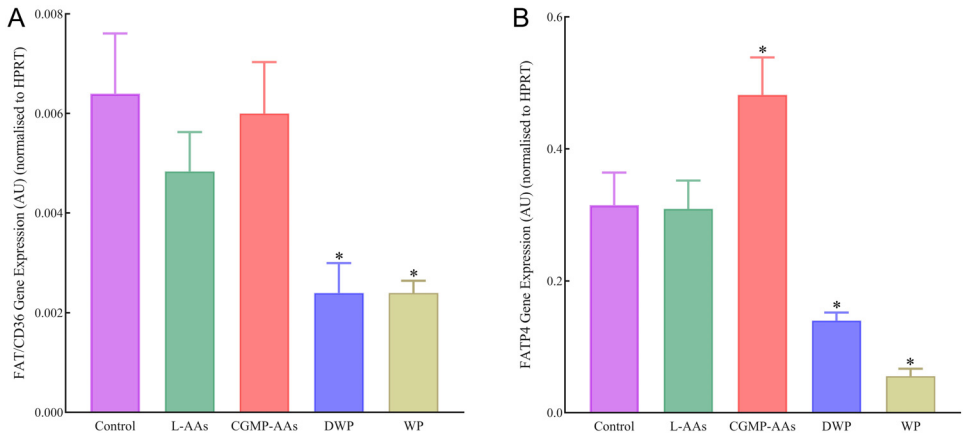

Fig. 8 – Chronic exposure (24 h) of Caco-2 cells to different protein substitutes (10 mg/mL) distinctly modulates fatty acid translocase (FAT)/ cluster of differentiation 36 (CD36) (A) and fatty acid transport protein 4 (FATP4) (B) gene expression. Relative expression of these genes was evaluated by quantitative RT-qPCR. Results were normalized for the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Data are shown as mean ± SEM (n = 5-6). *P < .05 vs. control (one-way ANOVA with post-hoc test). Abbreviations: AU - arbitrary unit, L-AAs - phenylalanine-free L-amino acid supplements, CGMP-AAs - casein glycomacropeptide with amino acids, DWP - digested whey protein, WP - whey protein.

PEPT1 expression. Amino acid mixtures deliver high concentrations of free amino acids to enterocytes, which are absorbed through multiple transport systems [44]. It is possible that some of these transporters may be downregulated to prevent excessive amino acids absorption. However, this potential downregulation did not affect FITC D-alanine absorption, likely because alanine is transported by other carriers different from LAT1 [35]. Moreover, the downregulation of PEPT1, a transporter of di- and tripeptides [45], may suggest a substrate-dependent regulation, where the transporter is downregulated in the absence of its substrate [46]. This could potentially be triggered by the exclusive presence of free amino acids as the nitrogen source in L-AAs formulations [3]. Regarding bacterial growth, L-AAs strongly promoted the growth of the three tested bacteria, likely due to their rapid bioavailability for microbial uptake [47]. Notably, *E. coli* propionate production increased following exposure to L–AAs. This finding contrasts with a mice study in which an amino acid–based diet, compared with a standard diet, was associated with reduced SCFA production (48).

Collectively, these findings suggest that L-AAs reduce glucose absorption in enterocytes, which could potentially have a positive impact on the regulation of plasma glucose levels, in addition to controlling Phe concentration. Moreover, L-AAs also serve as an easily metabolizable substrate that promotes the growth of both beneficial and opportunistic gut bacteria.

Regarding CGMP-AAs, this PS did not affect glucose and fatty acids absorption in Caco-2 cells. However, it significantly reduced L-alanine absorption and upregulated LAT1 gene expression. LAT1 primarily transports large neutral amino acids (LNAAs) - leucine, isoleucine, valine, phenylalanine, tyrosine, tryptophan, methionine, and histidine [48]. In this study, the

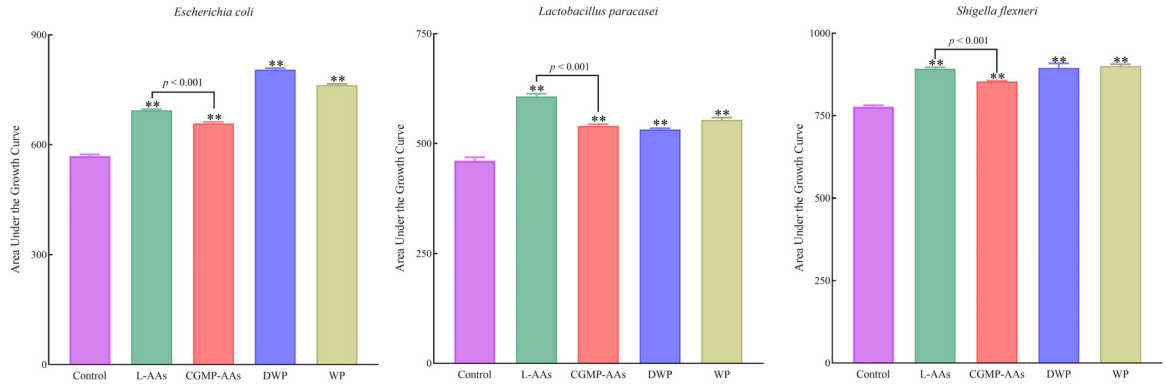

Fig. 9 – Exposure (24 h) to protein substitutes (0.3 mg/mL) increases bacterial final abundance, as determined by the area under the growth curve of *Escherichia coli*, *Lactobacillus paracasei*, and *Shigella flexneri*. Data shown as mean ± SEM (n = 9). **P < .001 vs. control (one-way ANOVA with post-hoc test). Abbreviations: L-AAs - phenylalanine-free L-amino acid supplements, CGMP-AAs - casein glycomacropeptide with amino acids, DWP - digested whey protein, WP - whey protein.

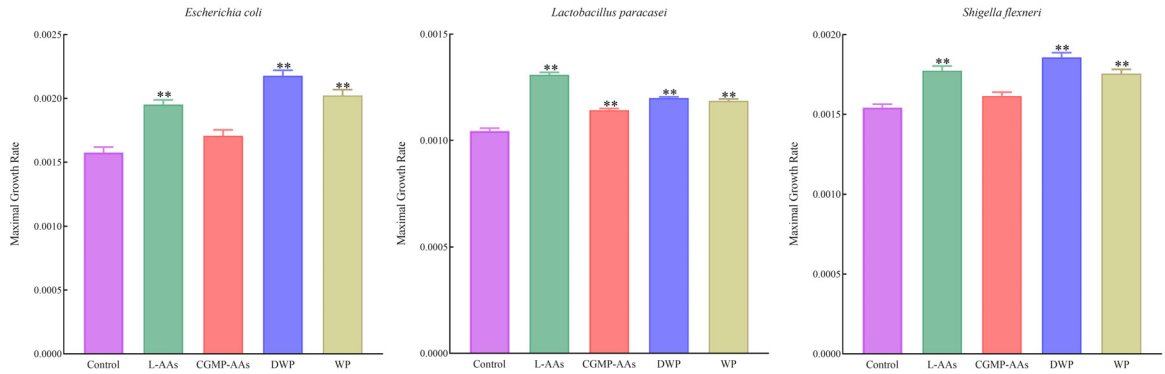

Fig. 10 – Exposure (24 h) to protein substitutes (0.3 mg/mL) increases bacterial growth rate, as determined by the bacterial maximal growth rate (r_{max}) of *Escherichia coli*, *Lactobacillus paracasei* and *Shigella flexneri*. Data shown as mean ± SEM (n = 9). **P < .001 vs. control (one-way ANOVA with post-hoc test). Abbreviations: L-AAs - phenylalanine-free L-amino acid supplements, CGMP-AAs - casein glycomacropeptide with amino acids, DWP - digested whey protein, WP - whey protein.

concentration of total LNAAs varied across the different protein formulations tested, with CGMP-AAs having the lowest overall concentration. Interestingly, clinical data support the idea that CGMP-AAs may enhance LNAA availability. Pena and colleagues [49] reported that patients consuming CGMP-AAs ingested similar amounts of tyrosine compared to those receiving L-AAs, yet exhibited significantly higher plasma tyrosine concentrations. Similarly, Daly et al. [50] observed that children with PKU consuming CGMP-AAs had higher plasma tyrosine concentrations than those receiving L-AAs. In patients adhering to a Phe-restricted diet, this could represent a clinical advantage: increased LAT1 expression may promote the absorption of LNAAs other than Phe, increasing their circulating levels. This, in turn, may reduce the preferential transport of Phe across the blood–brain barrier, potentially improving metabolic control and clinical outcomes in individuals with PKU [51].

Additionally, CGMP-AAs promoted bacterial growth although to a lesser extent than L-AAs. Given that *E. coli* and *L. paracasei* are known gas producers, primarily producing hydrogen and carbon dioxide [52,53], the lower bacterial proliferation with CGMP-AAs may result in less gas production. This observation could partially contribute to explain the improved gastrointestinal symptoms reported with CGMP consumption. In fact, Pinto et al. [54] observed that CGMP-AAs significantly alleviated several gastrointestinal symptoms, including wind and bloating compared to L-AAs. Additionally, there was a trend toward reduced constipation further supporting a potential gut-tolerability benefit of CGMP-based formulations. Interestingly, CGMP-AAs selectively increased the replication rate (R_{max}) of the probiotic strain *L. paracasei*, without affecting the growth of *S. flexneri* and *E. coli*, confirming that this PS affects bacterial growth in a strain-specific manner. Indeed, CGMP, the core component of CGMP-AAs, is known to have prebiotic properties, particularly due to its ability to promote the growth of *Bifidobacterium* and lactic acid bacteria [55]. Given that *L. paracasei* is a lactic acid bacterium [56], the findings of this study provide further support for the prebiotic potential of CGMP-containing formulations. By preferentially stimulating the proliferation of *L. paracasei* and similar beneficial bacterial species, CGMP-AAs may eventually contribute to the modulation of gut microbiota towards a more favourable composition, which could alleviate gastrointestinal symptoms [57]. However, this should be explored further using

more complex models that allow interactions between multiple bacterial species. Moreover, no meaningful differences were observed in SCFA or BCFA production in this study, a finding consistent with clinical data from a 6–month intervention in patients with PKU (5 children and 4 adults), in which CGMP–AAs supplementation did not significantly alter gut microbiota composition or SCFA concentrations [58].

Finally, the effects of WP and DWP on intestinal nutrient absorption were distinct from those observed with L-AAs and CGMP-AAs. This suggests that the impact of PS on intestinal nutrient absorption is specific rather than a generic response to free amino acids or small peptides. As DWP contains small peptides and free amino acids, one might have expected that L-AAs or CGMP-AAs would mimic its effects. However, this was not the case, reinforcing that the alterations induced by PS represent unique and formulation-specific effects.

Taken together, these results highlight, for the first time, the distinct effects of each PS on nutrient absorption, transporters expression and gut bacterial growth. These findings do not indicate that one protein substitute is universally superior to the other. Rather, they emphasize the importance of considering not only the nutritional adequacy of each PS, but also their potential broader metabolic effects, particularly in individuals with PKU.

Importantly, the concentrations of PS used in the experiments were selected to reflect postprandial protein concentrations typically found in the human jejunum after a protein-rich meal [31]. This approach supports the physiological relevance of the findings, while minimizing the risk of non-physiological effects. Nonetheless, this study has some limitations that should be acknowledged. While Caco-2 cells are a well-established *in vitro* model for human enterocytes, they do not fully replicate the complexity of the intestinal epithelium *in vivo*. Notably, this simplified *in vitro* system does not recapitulate the dynamic processes of gastrointestinal digestion, enteroendocrine and systemic hormonal regulation, or the complex interactions within the whole gut microbiota ecosystem. Additionally, these experiments were conducted under basal conditions. Inflammatory or oxidative stress *in vivo*, such as increased intestinal permeability, could potentially modulate nutrient transporter activity and microbial interactions. Future studies using coculture or inflammatory models would be valuable to further investigate these effects. Additionally, while protein and carbohydrate contents were matched across formulations, other constituents, such as lipids, vitamins and minerals, could also have contributed to the observed effects. With these considerations, these findings should be interpreted as preliminary evidence and a basis for future investigation. Further translational and clinical research is essential to confirm these results and to guide personalised and evidence-based prescription strategies for PS in patients with PKU.

5. Conclusion

This pioneering *in vitro* study demonstrates that PS distinctly modulate intestinal nutrient absorption and gut bacterial growth. These findings highlight the need to consider the potential metabolic responses following different PS intake in patients with PKU. Further translational and clinical research is essential to confirm these results, and guide evidence-based strategies for optimising PS use in this population.

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Author declarations

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CRediT authorship contribution statement

Catarina Rodrigues: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shámila Ismael:** Writing – review & editing, Investigation. **Ana Rita Monteiro:** Writing – review & editing, Investigation. **Maria João Almeida:** Writing – review & editing, Investigation. **Tiago Noronha:** Writing – review & editing, Investigation. **Gilberto Maia-Santos:** Writing – review & editing, Investigation. **Iva Fernandes:** Writing – review & editing, Investigation. **Conceição Calhau:** Writing – review & editing, Investigation. **Anita MacDonald:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Júlio César Rocha:** Writing – review & editing, Supervision, Investigation, Conceptualization. **João R. Araújo:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Ana Faria:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of AI and AI-assisted technologies

No AI or AI-assisted technologies were used during the preparation of this work.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nutres.2026.04.013.

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