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# Glycomacropeptide (GMP) rescued the oxidative and inflammatory activity of free L-AAs in human Caco-2 cells: New insights that support GMP as a valid and health-promoting product for the dietary management of phenylketonuria (PKU) patients

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ABSTRACT

Phenylketonuria represents the most prevalent inborn error of amino acid metabolism. In early diagnosed patients adequate and continued dietary treatment results in a good neurologic outcome. However, due to the natural protein and phenylalanine-restricted diet, oxidative stress represents a concern in phenylketonuric patients. Clear evidences suggest that the pathophysiology of PKU is also dependent by mitochondrial impairment and oxidative stress. In this context due to the tight connection between oxidative and inflammatory stress and noncommunicable diseases (NCDs) development, it is reasonable to hypothesize that PKU patients may present a higher risk to develop NCDs during their life. Currently available protein substitutes on the market include free amino acids (L-AAs), prolonged-release protein substitute and formula containing glycomacropeptide (GMP). Our results suggest that free L-AAs significanlty worsens the intestinal hydrogen peroxide ($H_2O_2$) and lipopolysaccharides (LPS)-induced oxidative and inflammatory status in Caco-2 cells, which are significantly restored towards physiological condition by GMP alone and when present in a 1:1 mixture with free L-AAs, providing new preclinical piece of information which can shed a shadow on the mechanism of action of these products on PKU patients and their future management.

## 1. Introduction

Hyperphenylalaninemia (HPA) is a disorder usually caused by the deficiency of the enzyme phenylalanine hydroxylase (PAH), which converts dietary phenylalanine (Phe) to tyrosine (Tyr) (Scriver & Kaufman, 2001; Wiederschain, 2002). The range of the disease severity observed among patients with this form of HPA is primarily due to allelic heterogeneity at the PAH locus. Various combinations of mutations result in a full spectrum of metabolic phenotypes ranging from severe, moderate, to mild phenylketonuria (PKU, blood Phe concentration > 360 mmol/L), which require dietary management, as well as mild hyperphenylalaninemia (MHP, blood Phe 120–360 mmol/L), in which dietary restriction is not necessary (van Spronsen et al., 2017). Untreated patients with PKU suffer from progressive and irreversible neurological impairment during childhood. Furthermore, behavioral abnormalities (es. hyperactivity, aggressiveness, anxiety) can cause severe social problems and are commonly seen in untreated PKU patients (Blau et al., 2010). Thanks to the introduction of neonatal screening, individuals affected by PKU are diagnosed earlier and can benefit of dietary treatment from the neonatal period onwards. PKU dietary treatment is mainly based on (Van Wegberg et al., 2017): 1) restriction of Phe intake by natural foods, 2) supplementation with a Phe-free

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amino acid mixtures to prevent protein deficiency, 3) consumption of Foods for Special Medical Purposes (FSMPs), and 4) supplementation of minerals, vitamins and other essential nutrients (Verduci et al., 2020). Protein rich foods such as dairy products, meat, fish, and eggs are usually excluded. Currently available protein substitutes on the market include free amino acids mixture (L-AAs), prolonged-release protein substitute and formula containing glycomacropeptide (GMP). Notably GMP is a peptide derived from the C-terminal part (f 106–169) of kappa-casein which is released in whey during cheese making by the action of chymosin (Neelima et al., 2013). GMP is significantly rich in the amino acids Pro, Glu, Ser, and Thr but depleted in Trp, Tyr, Phe, and Cys. The true 3D structure of GMP could not be established by crystallization, however, a theoretical model predicted that GMP is a random coil or intrinsically disordered peptide without a defined secondary or tertiary structure. In addition, recent evidence suggest that GMP exerts health-promoting activity (Neelima et al., 2013). In this context, many of the biological properties have been ascribed to the carbohydrate moieties (i.e. inhibition of cholera toxin, modulation of immune response, and prevention of intestinal infection) which are sialic acid residues, attached to the peptide (Isoda et al., 1992; Nakajima et al., 2005; Requena et al., 2010), some other are due to the peptides backbone (i.e. antibacterial and probacterial activity of pepsin-treated GMP, anti-cariogenic effect, and bifido factor) (Aimutis, 2004; Azuma et al., 1984; Robitaille et al., 2012). Recently literature indicated that GMP displays hypocholesterolemic and anti-diabetic activity in both *in vitro* on human hepatic HepG2 cells and *in vivo* in animal models (Sauvé, Spahis, et al., 2021). Moreover a recent study on the gut microbiota of PKU patients showed that 6 months of GMP supplementation is not only clinically and microbiologically safe, but also ameliorates calcium phosphate homeostasis, with increasing values of vitamin D, suggesting a possible beneficial indirect effect on bone health (Montanari et al., 2022).

In addition, since it contains residual amounts of aromatic amino acid as Phe (Daly et al., 2021), GMP is considered a useful product for the dietary management of PKU patients, although, up to date evidence on GMP in PKU is still limited, therefore it cannot be considered a well-established alternative in children or pregnancy (Van Wegberg et al., 2017). As GMP has inadequate amounts of histidine, leucine, tryptophan, methionine and tyrosine, commercial GMP products are enriched with these limiting and indispensable amino acids (van Calcar & Ney, 2012). Indeed, among available protein substitutes, free L-AAs, which are characterized by a rapid absorption after ingestion, which in turn leads to a peak and a consequent quick decrease in plasma AAs levels, are used. Despite literature reports that AAs have some protective effects on tissue oxidative stress in human intestinal epithelial cells (Katayama & Mine, 2007), the consumption of free-AAs is associated with more AA oxidation, higher levels of blood urea nitrogen (BUN) and less protein accretion compared to slowly digested proteins in healthy individuals; the faster absorption of free AAs compared to AAs from whole proteins is also associated with less efficient utilization of the same AAs and effects on glycemic control (Scheinin et al., 2020, 2021). In principle, free L-AAs are absorbed at a different rate than natural dietary proteins and in general, foods are not characterized for the presence of free L-AAs. Hence, the exposition of the intestine to free L-AAs is unphysiologically which can lead to oxidative and inflammatory status, which can significantly affect the pathophysiological progression of PKU leading to an increase of the risk of developing non-communicable diseases (NCDs). Treatment for IEMs very often requires long-term restricted diets, which have shown to promote a state of dysbiosis and alterations in the composition of the gut microbiota. For this reason, systemic pro-inflammatiory status due to modified microbiota profile is potentially linked to a higher risk of NCDs development (Montanari et al., 2021).

In light with our hypothesis, the present study is aimed at investigating the effect of free L-AAs and GMP on human intestinal Caco-2 cells. In particular, we prevented bias in the examination of the impact on oxidative to inflammatory stress by selecting a Docosahexaenoic acid (DHA)-free AA mixture. More in details, $H_2O_2$ and lipopolysaccharides (LPS) were used as stimuli for mimicking the oxidative and inflammatory stress, respectively and both free L-AAs and GMP were tested in order to assess their ability to restore a more physiological condition. In parallel, a mixture of free L-AAs and GMP (1:1) was also characterized, since PKU patients usually consume 50% of GMP-derived protein and 50% of AA mixture as protein source within their diet (Montanari et al., 2022). Dedicated experiments were performed in order to evaluate their direct antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) assays, respectively. Afterwards, the ability of each tested sample to modulate the reactive oxygen species, lipid peroxidation and nitric oxide (NO) production as well as the production of pro- and anti-inflammatory cytokines were monitored in human intestinal Caco-2 cells, providing new preclinical piece of information which can shed a shadow on the mechanism of action of these products on PKU patients and their future management.

## 2. Materials and methods

### 2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin/streptomycin, chemiluminescent reagent, and 24 or 96-well plates were purchased from Euroclone (Milan, Italy). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], DPPH (1,1-diphenyl-2-picrylhydrazyl), TPTZ, Griess reagent, bovine serum albumin (BSA), RIPA buffer, the antibody against β-actin, fluorometric intracellular ROS kit and MDA assay kit were bought from Sigma-Aldrich (St. Louis, MO, USA). Phenylmethanesulfonyl fluoride (PMSF), Na-orthovanadate inhibitors, and the antibodies against rabbit Ig-horseradish peroxidase (HRP) and mouse Ig-HRP were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The inducible Nitric Oxide Synthase (iNOS) primary antibody came from Cell Signaling Technology (Danvers, MA, USA); the inhibitor cocktail Complete Midi from Roche (Basel, Swiss); Mini protean TGX pre-cast gel 7.5% and Mini nitrocellulose Transfer Packs from BioRad (Hercules, CA, USA). The nutritional composition of free L-AAs mixture and GMP used in the experiments are reported in supplementary tables.

### 2.2. DPPH (2,2-diphenyl-1-picrylhydrazyl radical scavenging) assay

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH) assay was performed to determine the antioxidant activity by standard method with a slight modification. Briefly, the DPPH solution (12.5 μM in methanol, 45 μL) was added to 15 μL of GMP, L-AAs and the 1:1 mixture at different concentrations (1 – 20.0 mg/mL) in a 96-well half area plate. The reaction for scavenging DPPH radicals was performed in the dark at room temperature and the absorbance was measured at 520 nm after 30 min incubation.

### 2.3. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay evaluates the ability of a sample to reduce ferric ion ($Fe^{3+}$) into ferrous ion ($Fe^{2+}$). Thus, 10 μL (15X) of GMP, L-AAs and the 1:1 mixture were mixed with 140 μL of FRAP reagent. The FRAP reagent was prepared by mixing 1.3 mL of a 10 mM TPTZ (Sigma-Aldrich, Milan, Italy) solution in 40 mM HCl, 1.3 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 13 mL of 0.3 M acetate buffer (pH 3.6). The microplate was incubated for 30 min at 37 °C and the absorbance was read at 595 nm. Absorbances were recorded on a Synergy™ HT-multimode microplate reader.

### 2.4. Cell culture

Caco-2 cells, obtained from INSERM (Paris, France), were routinely

sub-cultured at 50% density and maintained at 37 ∘C in a 90% air/10% $CO_2$ atmosphere in DMEM containing 25 mM of glucose, 3.7 g/L of NaHCO3, 4 mM of stable L-glutamine, 1% nonessential amino acids, 100 U/L of penicillin, and 100 μg/L of streptomycin (complete medium), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA).

### 2.5. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

A total of $3 \times 10^4$ Caco-2 cells/well were seeded in 96-well plates and treated with GMP, L-AAs and the 1:1 mixture from 0.1 to 50.0 mg/mL, or vehicle, in complete growth media for 48 h at 37 °C under 5% $CO_2$ atmosphere. Subsequently, the treatment solvent was aspirated and 100 μL/well of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) filtered solution added. After 2 h of incubation at 37 °C under 5% $CO_2$ atmosphere, 0.5 mg/mL solution was aspirated and 100 μL/well of the lysis buffer (8 mM HCl + 0.5% NP-40 in DMSO) added. After 10 min of slow shaking, the absorbance at 575 nm was read on the Synergy H1 fluorescence plate reader (Biotek, Bad Friedrichshall, Germany).

### 2.6. Fluorometric intracellular ROS assay

For cells preparation, $3 \times 10^4$ Caco-2 cells/well were seeded on a black 96-well plate overnight in growth medium. The day after, the medium was removed and replaced with 50 μL/well of the Master Reaction Mix and the cells were incubated at 5% $CO_2$, 37 °C for 1 h in the dark. Then, cells were treated with 5 μL of 11X GMP, L-AAs and the 1:1 mixture (to reach the final concentrations of 1.0, 5.0 and 10.0 mg/mL) and incubated at 37 °C for 24 h in the dark. To induce ROS, cells were treated with 5 μL of $H_2O_2$ at a final concentration of 1.0 mM for 60 min a 37 °C in the dark and fluorescence signals (ex./ em. 490/525 nm) were recorded using a Synergy H1 microplate reader.

### 2.7. Lipid peroxidation (MDA) assay

Caco-2 cells ($2.5 \times 10^5$ cells/well) were seeded in a 24 well plate and, the following day, they were treated with GMP, L-AAs and the 1:1 mixture (at the final concentrations of 5.0 and 10.0 mg/mL) for 24 h at 37 °C under 5% $CO_2$ atmosphere. After incubation, cells were stimulated with $H_2O_2$ 1 mM, or vehicle, for 60 min, then collected and homogenized in 150 μL ice-cold MDA lysis buffer containing 1.5 μL of BHT (100X). Samples were centrifuged at 13,000 × g for 10 min, then they were filtered through a 0.2 μm filter to remove insoluble material. To form the MDA-TBA adduct, 300 μL of the TBA solution were added into each vial containing samples and incubated at 95 °C for 60 min, then cooled to RT for 10 min in an ice bath. For analysis, 100 μL of each reaction mixture were pipetted into a 96 well plate and the absorbance was measured at 532 nm using the Synergy H1 fluorescent plate reader from Biotek.

### 2.8. Nitric oxide level evaluation on Caco-2 cells

Caco-2 cells ($1.5 \times 10^5$/well) were seeded on 24-well plates. The next day, cells were treated for 24 h with GMP, L-AAs and the 1:1 mixture to reach the final concentrations of 10.0 mg/mL and incubated at 37 °C under a 5% $CO_2$ atmosphere. After incubation, cells were stimulated with $H_2O_2$ (1.0 mM) or vehicle for 1 h, then the cell culture media were collected and centrifuged at 13,000 × g for 15 min to remove insoluble material. NO determination was carried out by Griess test. Briefly, 1.0 g of Griess reagent powder were solved in 25.0 mL of distilled $H_2O$ and 50.0 μL of the solution were incubated with 50.0 μL of the culture supernatants for 15 min at RT in the dark. The absorbance was measured at 540 nm using the Synergy H1 fluorescent plate reader from Biotek.

### 2.9. Western blot analysis

$1.5 \times 10^5$ Caco-2 cells/well (24-well plate) were treated with 10.0 mg/mL of GMP, L-AAs and the 1:1 mixture sample for 24 h. After incubation, the cells were stimulated with $H_2O_2$ (1.0 mM) or LPS (1 μg/mL) or vehicle for 24 h, then the cell culture media were collected in an ice-cold microcentrifuge tube and processed for the Griess assay and for the cytokines quantifications. Meanwhile the cells were scraped in 30 μL ice-cold lysis buffer [RIPA buffer + inhibitor cocktail + 1:100 PMSF + 1:100Na-orthovanadate] and transferred in an ice-cold microcentrifuge tube. After centrifugation at 16,060 g for 15 min at 4 °C, the supernatant was recovered and transferred in a new ice-cold tube. Total proteins were quantified by the Bradford method and 50 μg of total proteins loaded on a pre-cast 7.5% sodium dodecyl sulphate - polyacrylamide (SDS-PAGE) gel at 130 V for 45 min. Subsequently, the gel was pre-equilibrated with 0.04% SDS in $H_2O$ for 15 min at room temperature (RT) and transferred to a nitrocellulose membrane (Mini nitrocellulose Transfer Packs), using a Trans-blot Turbo at 1.3 A, 25 V for 7 min. Target proteins, on milk or BSA blocked membrane, were detected by primary antibodies as follows: anti-iNos and anti-β-actin. Secondary antibodies conjugated with HRP and a chemiluminescent reagent were used to visualize target proteins and their signal was quantified using the Image Lab Software (Biorad). The internal control β-actin was used to normalize loading variations.

### 2.10. Pro-inflammatory and anti-inflammatory secreted cytokines quantification

Cytokines quantification was performed using human Qunatikine® ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instruction. Briefly, the supernatants collected from treated and LPS-stimulated Caco-2 cells were centrifuged at 13,300 × g for 10 min at 4 °C, then the pellet and insoluble material were discarded. For the experiments, 100 μL of samples were added to each well and the microplate was incubated for 2 h at room temperature (RT). After the incubation, the solutions were discarded and each well washed 4 times with 300 μL of Wash Buffer solution, then 200 μL of Conjugate solution were added to each well and the microplate was incubated for 2 h at room temperature (RT). After incubation, the solutions were discarded and each well washed 4 times with 300 μL of Wash Buffer solution; than 200 μL of Substrate Solution were added and the plate was incubated for 1–4 h at RT in the dark. The reactions were stopped with 50 μL of Stop Solution and the absorbance at 450 and 540 nm was measured using the Synergy H1 plate reader (BioTek Instruments).

### 2.11. Statistical analysis

All results were expressed as the mean ± standard deviation (s.d.), where p-values $< 0.05$ were considered to be significant. All the data sets were checked for normal distribution by D'Agostino and Pearson test. Since they are all normally distributed with p- values $< 0.05$, statistical analyses were performed by one- way ANOVA followed by Tukey's post-test, respectively (Graphpad Prism 9, GraphPad Software, La Jolla, CA, USA).

## 3. Results

### 3.1. In vitro evaluation of the free L-AAs and GMP radical scavenging activity by DPPH and FRAP assays

In order to assess the antioxidant properties of free L-AAs and GMP, the DPPH and FRAP assays were performed. As reported in Fig. 1A, results suggest that L-AAs scavenged the DPPH radical by 9.26 ± 8.18%, 11.49 ± 8.3% and 8.75 ± 19.36% at 1.0, 10.0 and 20.0 mg/mL, respectively. GMP reduced the DPPH radical by 9.95 ± 1.34%, 39.53 ± 4.82 % and 61.0 ± 8.85 % at 0.0, 10.0 and 20.0 mg/mL, respectively

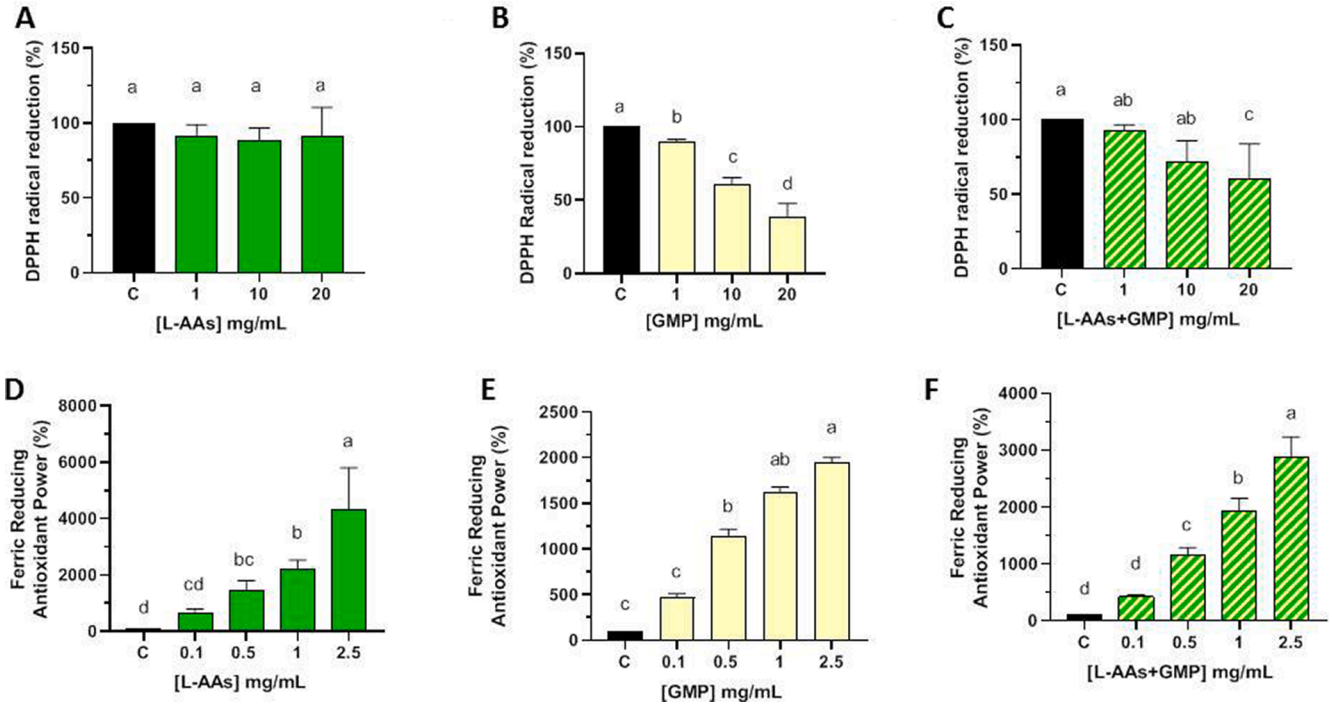


**Fig. 1.** In vitro radical scavenging activity of free L-AAs, GMP and the mixture on DPPH (A-C) and FRAP (D-F) assays, respectively. Data represent the mean ± s.d. of six determinations performed in triplicate. All the data sets have been analyzed by One-way ANOVA followed by Tukey's post-hoc test. Different lowercase letters indicate a significant difference ($p < 0.05$) between different concentrations. C: control,

(Fig. 1B). Finally, the 1:1 mixture scavenged the DPPH radical by 7.03 ± 3.6%, 28.19 ± 14.13 % and 39.62 ± 23.29 % at 0.0, 10.0 and 20.0 mg/mL, respectively, tested at the same concentrations (Fig. 1C).

Moreover, the free L-AAs increased the FRAP by 663.5 ± 129 %, 1450 ± 347.9 %, 2192 ± 343.9% and 4373 ± 1469 % at 0.1, 0.5, 1.0 and 2.5 mg/mL, respectively (Fig. 1D). Fig. 1E shows that GMP increased the FRAP power by 474.2 ± 37.06 %, 1132 ± 845.2 %, 1626 ± 547.4% and 1948 ± 547.4 % at 0.1, 0.5, 1.0 and 2.5 mg/mL, respectively (Fig. 1E). Finally, the 1:1 mixture is able to augment the FRAP by 422.6 ± 33.03 %, 1164 ± 124.3 %, 1928 ± 227.2% and 2891 ± 343.6%, respectively, tested at the same concentrations (Fig. 1F).

### 3.2. Evaluation of free L-AAs and GMP effects on the Caco-2 cells viability

On the basis of the previously obtained results, the cellular evaluations of free L-AAs and GMP antioxidant properties were carried out. Before proceeding to experiments on Caco-2 cells, it was necessary to perform the MTT experiments for verifying that the compound did not impair the cellular vitality. Results suggested that free L-AAs sample, alone and in the mixture, was safe for intestinal cells from 0.1 to 10 mg/mL, whereas GMP was safe at all the concentrations tested in the range 0.1 – 50 mg/mL (Fig. 2). Free L-AAs treatment reduced the Caco-2 cell viability by 55.8 ± 2.5% compared to the control, whereas GMP is completely safe for the Caco-2 cells in the range 0.1–50 mg/mL. The 1:1 mixture free L-AAs /GMP reduced the intestinal cell vitality by 40 ± 6.5% at 50 mg/mL. These results clearly suggest that GMP is able to preserve the Caco-2 cellular vitality confirming that is a safer product than free L-AAs.

### 3.3. Effect assessment of free L-AAs and GMP on the $H_2O_2$-induced ROS and lipid peroxidation levels in human intestinal Caco-2 cells

To evaluate the ability of free L-AAs and GMP to vary ROS overproduction induced by $H_2O_2$, cellular experiments were carried out. Our findings clearly demonstrated that Caco-2 cells treated with $H_2O_2$ alone showed an increase of ROS levels up 411.4 ± 13.16%, versus the control cells. Fig. 3A shows that free L-AAs increased the intracellular $H_2O_2$-

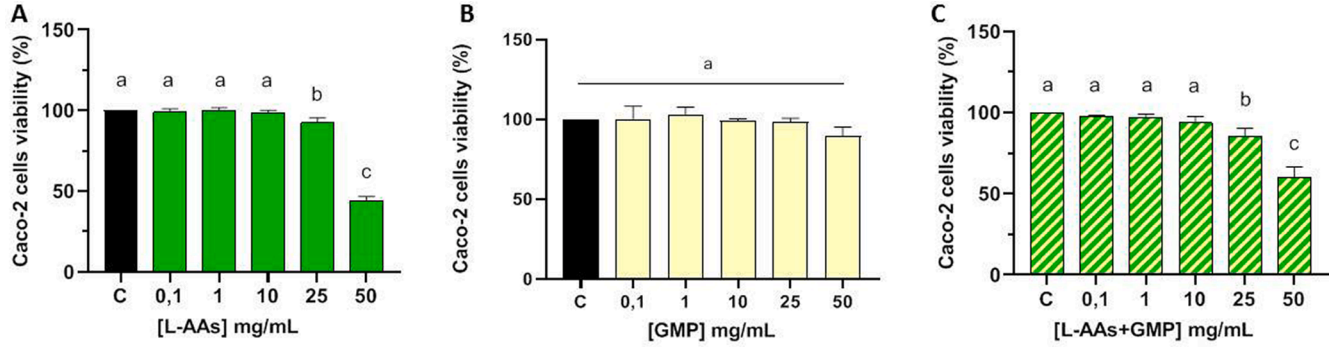


**Fig. 2.** Effects of free L-AAs (A), GMP (B), and free L-AAs/GMP mixture (C) on human intestinal Caco-2 cells. Data represent the mean ± s.d. of six determinations performed in triplicate. Different lowercase letters indicate a significant difference ($p < 0.05$) between different concentrations. C: control,

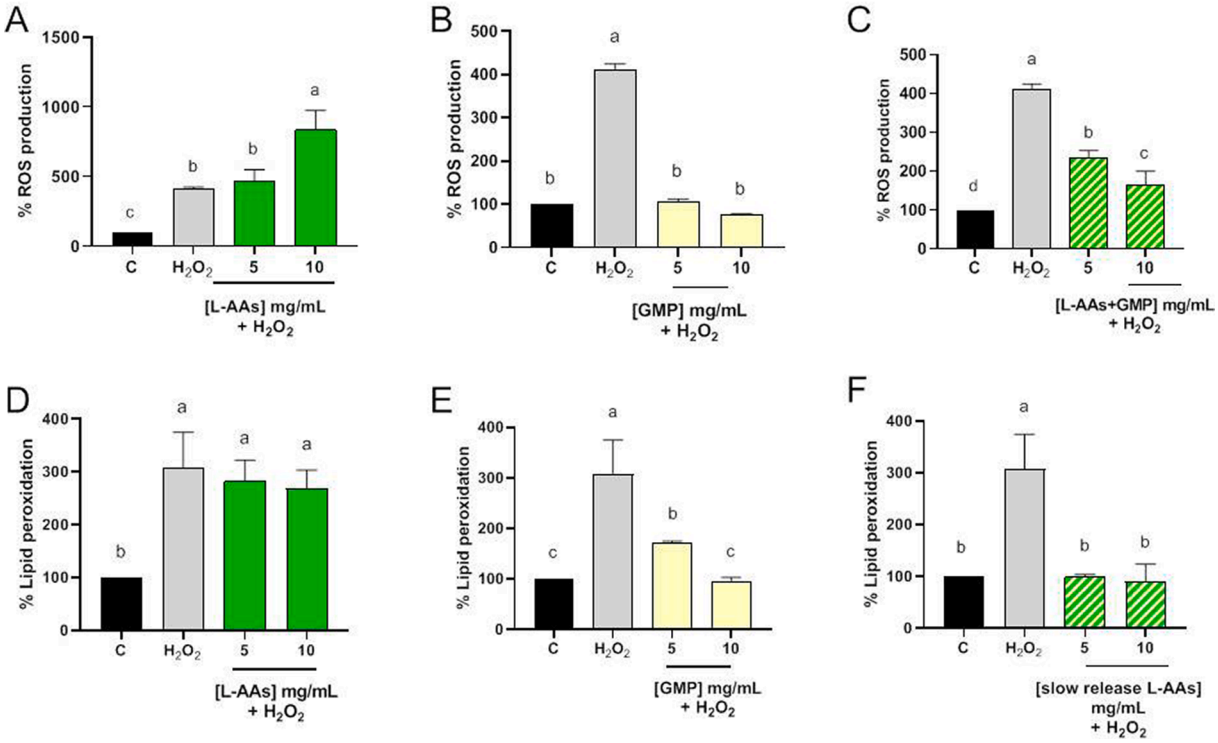


**Fig. 3.** Effect of free L-AAs (A-D), GMP (B-E), and free L-AAs/GMP mixture (C-F) on the modulation of $H_2O_2$-induced ROS and lipid peroxidation, respectively, in human intestinal Caco-2 cells. Data represent the mean ± s.d. of six determinations performed in triplicate. All the data sets have been analyzed by One-way ANOVA followed by Tukey's post-hoc test. Different lowercase letters indicate a significant difference ($p < 0.05$) between different concentrations. C: control,

induced ROS levels up to 431.4 ± 74.22% and 833.5 ± 141.4 at 5.0 and 10.0 mg/ml, respectively (Fig. 3A). On the contrary, Fig. 3B clearly shows that GMP decreased the ROS levels by 104,7 ± 7,67% and 74,29 ± 3,7%, respectively, tested at the same concentrations. In parallel, the 1:1 free L-AAs/GMP mixture is able to reduce the intracellular $H_2O_2$-induced ROS levels by 234,0 ± 7,61% and 165,1 ± 34,70%, tested at the same concentrations, respectively (Fig. 3C).

In addition, for evaluating the capacity of L-AAs and GMP to modulate the $H_2O_2$-induced lipid peroxidation in human intestinal Caco-2 cells, the MDA measurement was assessed. In agreement with the improvement of ROS after the same treatment with $H_2O_2$, a noticeable increase of the intracellular lipid peroxidation was detected up to 307,3 ± 67,62% versus the control cells. In addition, our results showed that the pretreatment with free L-AAs resulted in a modulation of MDA levels up to 282,0 ± 39,80%, and 268,1 ± 35,10%, at 5.0 and 10.0 mg/mL respectively (Fig. 3D). Contrary, the pretreatment with GMP reduced MDA levels up to 171,4 ± 3,31% and 94,73 ± 8,36 %, at 5.0 and 10.0 mg/mL, respectively (Fig. 3E). In parallel, the presence of GMP in the mixture positively contributed to decrease the MDA levels until 98,35 ± 5,332 % and 90,26 ± 33,48% at the same concentrations, respectively (Fig. 3F).

### *3.4. Effects of free L-AAs and GMP on the $H_2O_2$ and LPS-induced NO level production modulation via iNOS activation in Caco-2 cells*

The effects of -AAs and GMP on the production of NO levels were evaluated on human intestinal Caco-2 cells after oxidative stress induction. Notably, $H_2O_2$ (1 mM) treatment induced an oxidative stress that led to an increase of intracellular NO levels up to 143,9 ± 20,81% (Fig. 4). Pre-treatment with free L-AAs increased the $H_2O_2$-induced NO overproduction up to 153,7 ± 9,516%, at 10.0 mg/mL (Fig. 4A). On the contrary, pre-treatment with the mixture and GMP alone reduced the NO production up to 113,9 ± 5,21% and by 88.89 ± 5.5%, respectively, tested at the same concentrations (Fig. 4A-B).

In parallel, the effects free L-AAs and GMP on iNOS protein levels was assessed after oxidative stress induction by western blot experiments, in which the iNOS protein band at 130 kDa was detected and quantified. Results (Fig. 4 C-D) clearly demonstrated that, after $H_2O_2$ treatment (1 mM), the iNOS protein increased up to 136.2 ± 3.89%. Pre-treatment with L-AAs modulate the $H_2O_2$-induced iNOS level up to 127,7 ± 9,67 % at 10.0 mg/mL, versus control cells, while, the mixture reduced the $H_2O_2$-induced iNOS protein level up to 94.23 ± 3,23% at 10.0 mg/mL (Fig. 4C) and, in parallel, GMP decreased the $H_2O_2$-induced iNOS level up to 116,2 ± 3,9% at the same concentration (Fig. 4D).

In agreement with these results, LPS stimulation induced an inflammatory state in intestinal Caco-2 cells, increasing the the NO levels up to 143,3 ± 5,365% (Fig. 5 A-B) and the iNOS level productions up to 161,5 ± 21.8% ($p \leq 0.001$) (Fig. 5 C, D). The treatment with free L-AAs showed an augmentation in NO production up to 148,5 ± 11,10% (Fig. 5A) and iNOS production up to 177,1 ± 29,84% (Fig. 5C) and at 10.0 mg/mL. Otherwise, pre-treatment with the 1:1 mixture reduced the NO production up to 107,0 ± 3,075% and the iNOS production by 18.45 ± 6,644% (Fig. 5A, C), respectively, at the same concentration. In accordance, GMP significantly decreased the NO overproduction by 7.94 ± 4,49% and reduce the iNOS protein levels by 29.34 ± 25,14%, tested at 10.0 mg/mL (Fig. 5 B-D).

### *3.5. Investigation of the free L-AAs and GMP effects on the modulation of the LPS-Induced cytokine production in human intestinal cells*

To assess the pro- and anti-inflammatory activity of L-AAs and GMP, respectively, the effects of samples pre-treatment on the production of pro-inflammatory (IL-1β, IL-6, IFN-γ, and TNF- α) and anti-inflammatory (IL10) cytokines was determined in LPS-stimulated Caco-2 cell culture supernatants. As shown in Fig. 6, the LPS stimulation increased the production of the pro-inflammatory cytokines, and reduced the IL-10 production, compared with LPS-unstimulated cells (untreated control, C). More in details, LPS induced the TNF- α, IFN-γ, IL-1β and IL-6

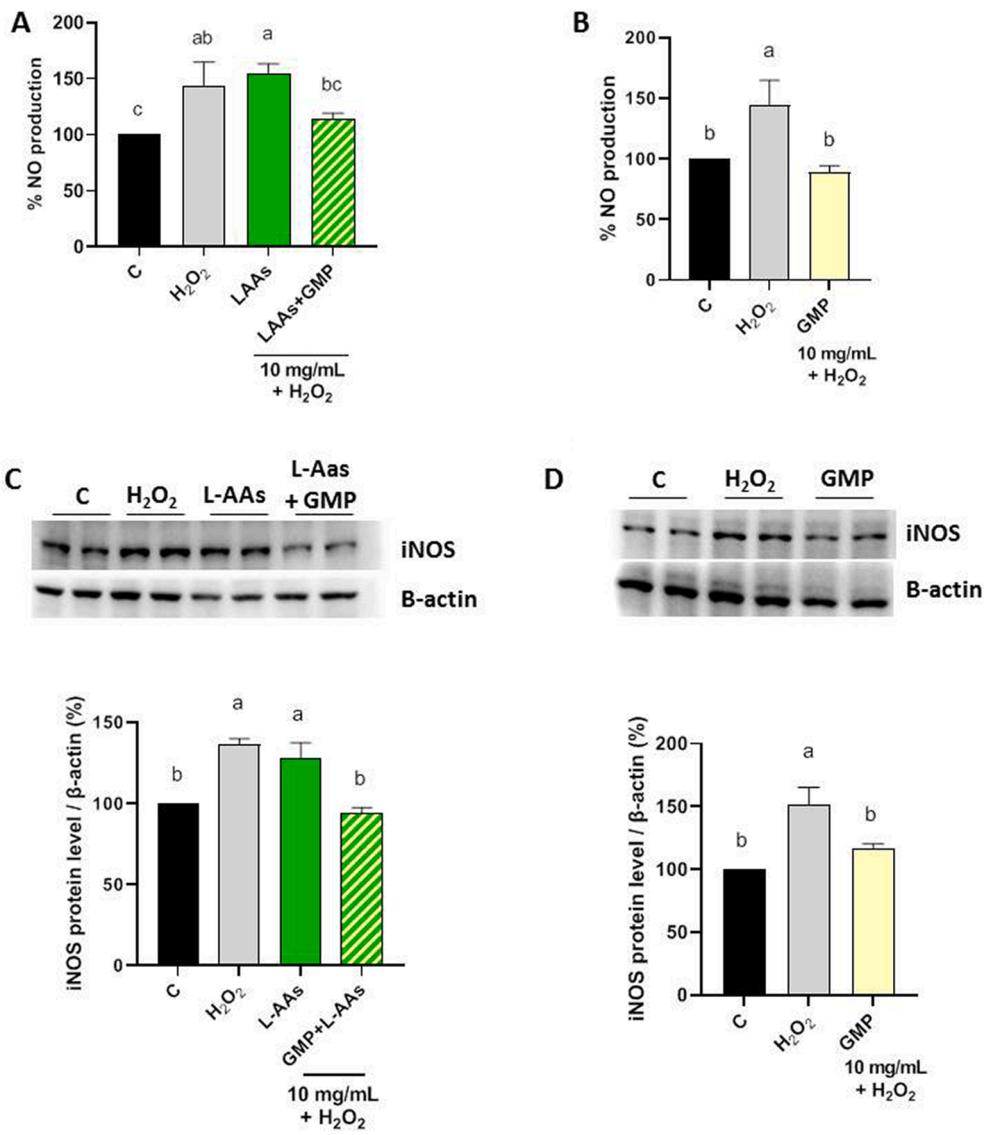


**Fig. 4.** Effect of free L-AAs, 1:1 free L-AAs/GMP mixture (A-C), and GMP (B-D) on the modulation of $H_2O_2$-induced NO production and iNOS protein levels, respectively, in human intestinal Caco-2 cells. Data represent the mean ± s.d. of six determinations performed in triplicate. All the data sets have been analyzed by One-way ANOVA followed by Tukey's post-hoc test. Different lowercase letters indicate a significant difference ($p < 0.05$) between different concentrations. C: control,

secretion up to 118.9 ± 1.48%, 165.6 ± 9.47%, 143.3 ± 2.88% and 158.0 ± 7.23% respectively, and reduced the IL-10 production by 5.95 ± 1.68% (Fig. 6). The L-AAs is unable to decrease the pro-inflammatory cytokines; in fact it increase the TNF- α up to 118.9 ± 1.48%; the IFN-γ is effectively augmented up to 668.7 ± 108.9%, whereas the IL-1β and IL-6 increase up to 138.6 ± 3.9% and 170.0 ± 14.14%, respectively, while the anti-inflammatory IL-10 is not modulated (Fig. 6A-E). Otherwise, the pretreatment with the 1:1 mixture, in which GMP is present, significantly reduced the TNF- α up to 103.8 ± 4.64%; the IFN-γ up to 218.8 ± 31.55%, the IL-1β and IL-6 up to 104.1 ± 5.7% and 115.0 ± 12.38%, respectively, while the anti-inflammatory IL-10 is augmented until the basal (control) value (Fig. 6A-E). In accordance, GMP effectively reduces the TNF- α up to 104.3 ± 8.11%; the IFN-γ up to 128.0 ± 7.5%, the IL-1β and IL-6 up to 132.1 ± 6.48% and 140.1 ± 2.9%, respectively, while the anti-inflammatory IL-10 is augmented up to 102.2 ± 3.99% (Fig. 6F-J).

## 4. Discussions

Early dietary treatment of PKU prevents severe neurological damage and generally leads to normal cognitive development, but subtle neurological deficits may persist. Clear evidences suggest that the pathophysiology of PKU is dependent by mitochondrial impairment and oxidative stress (Rocha & Martins, 2012). In this context due to the tight connection between oxidative and inflammatory stress to NCDs development (i.e. diabetes, hypertension, and cardiovascular disease) (Seyedsadjadi & Grant, 2021), it is reasonable to hypothesize that the PKU patients may present a higher risk over the time to develop NCDs. In this context, the disruption of pro-oxidant/antioxidant balance in phenylketonuric patients is strongly related to their dietary treatment.

As PKU dietary treatment requires early and life-long Phe-restricted diet, several studies have explored its possible relationship with cardiovascular risk factors (Verduci et al., 2016), because of the role of oxidative stress in cardiovascular diseases development. Results showed that PKU patients compliant with supplemented and balanced diets are not at risk for NCDs development but, at this time, there are inconsistent results in humans about oxidative stress due to PKU special diet.

In this context, our findings clearly indicate that the consumption of free L-AAs significantly worsen the intestinal oxidative and inflammatory status and that the GMP as well as the 1:1 free L-AA/GMP mixture positively restore the redox state and significantly ameliorate the inflammatory status. More in details, results indicate that free L-AAs are ineffective in scavenging the DPPH radicals, whereas GMP remove the DPPH radicals with a dose response trend, reaching 61.0 ± 8.85 % at 20.0 mg/mL, and in the 1:1 L-AAs/GMP mixture it significantly contribute to scavenge the DPPH radicals until 39.62 ± 23.29 % at the highest concentration tested (Fig. 1C).

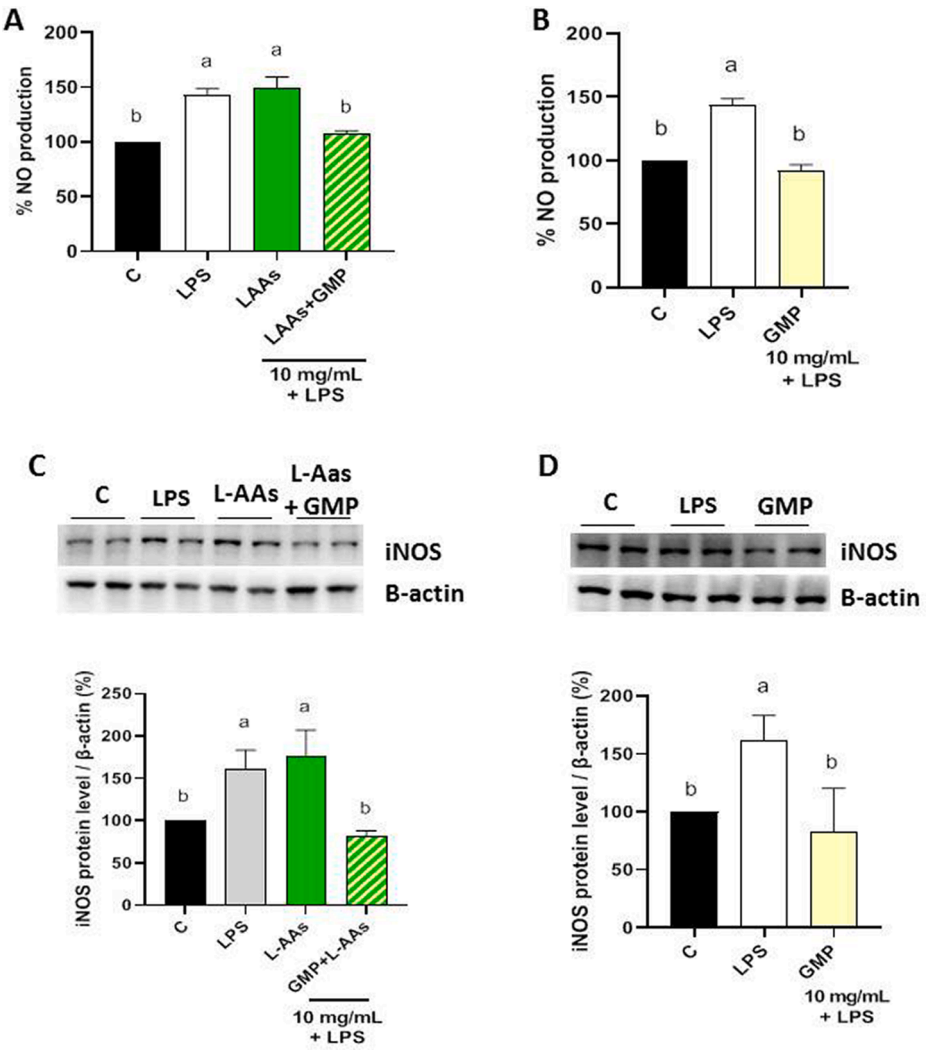


**Fig. 5.** Effect of free L-AAs, 1:1 free L-AAs/GMP mixture (A-C), and GMP (B-D) on the modulation of LPS-induced NO production and iNOS protein levels, respectively, in human intestinal Caco-2 cells. Data represent the mean ± s.d. of six determinations performed in triplicate. All the data sets have been analyzed by One-way ANOVA followed by Tukey's post-hoc test. Different lowercase letters indicate a significant difference ($p < 0.05$) between different concentrations. C: control,

Moreover, all the tested samples display FRAP activity. Based on these results and considering that intestine is the first barrier with which these dietary products come firstly in contact, a deep investigation has been performed at cellular level. Notably, human Caco-2 cells have been used as intestinal model and preliminary MTT experiments demonstrated that free L-AAs treatment reduced the Caco-2 cell viability by 55.8 ± 2.5% compared to the control, whereas GMP is completely safe for the Caco-2 cells in the range 0.1–50 mg/mL. The 1:1 mixture free L-AAs /GMP reduced the intestinal cell vitality by 40 ± 6.5% at 50 mg/mL. These results clearly suggest that GMP is able to preserve the Caco-2 cellular vitality confirming that is a safer product than free L-AAs (Fig. 2).

Taking into account these results, unlike free L-AAs, GMP restore the intracellular physiological levels of $H_2O_2$-induced ROS in Caco-2 cells at 5 and 10 mg/mL, respectively. In particular, it was observed that free L-AAs significantly improved the intracellular $H_2O_2$-induced ROS up to 431.4 ± 74.22% and 833.5 ± 141.4 at 5.0 and 10.0 mg/ml, respectively, and that GMP in the 1:1 mixture with free L-AAs, ameliorated their pro-oxidant effects, decreasing the ROS production by 234,0 ± 7,61% and 165,1 ± 34,70%, confirming its antioxidant activity at 5 and 10 mg/mL, respectively (Fig. 3 A-C).

In agreement with the ROS modulation, free L-AAs are ineffective in restoring the $H_2O_2$-induced lipid peroxidation in Caco-2 cells, whereas, in the same cellular system and concentrations, GMP positively modulated toward basal conditions the $H_2O_2$-induced lipid peroxidation and significantly counteracted the free L-AA pro-oxidant effects on $H_2O_2$-induced lipid peroxidation (Fig. 3 D-F). These results are in line with previous evidences which suggest that GMP mitigated oxidative stress, inflammation, lipoprotein biogenesis as well as improving insulin sensitivity and to favorably modulate the microbiota on intestinal Caco-2 cells (Foisy-Sauvé et al., 2020; Sauvé, Feldman, et al., 2021).

In addition, unlike the free L-AA sample, both alone and in the mixture with free L-AAs, GMP restored the physiological $H_2O_2$ - and LPS-induced NO production levels due to a direct effect on iNOS protein levels in CaCo-2 cells (Figs. 4 and 5). These results clearly suggested that NO play an important role in the cross link between oxidant and inflammatory status. Indeed, our finding demonstrated that GMP exert an anti-inflammatory activity due to the reduction of LPS-induced pro-inflammatory cytokines (IFN-γ, TNF- α, IL-1β, and IL-6) levels and an improvement of the LPS-reduced anti-inflammatory cytokine (IL-10) levels toward physiological conditions, respectively. On the contrary, free L-AAs significanlty worsen the LPS-induced IFN-γ and IL6 and it is ineffective in the improvement of LPS-reduced IL10 levels. This pro-inflammatory behavior of free L-AAs is marked counteracted by GMP

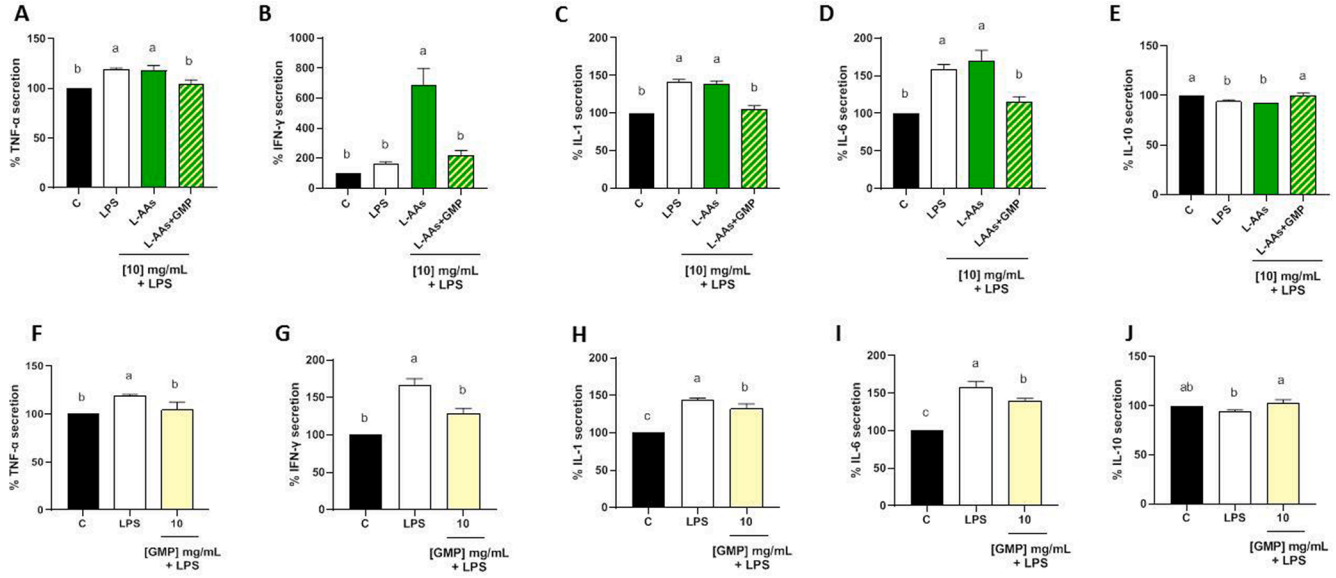


**Fig. 6.** Assessment of free L-AAs and 1:1 free L-AAs/GMP mixture on LPS-induced pro-inflammatory cytokines TNF- α (A), IFN-γ (B), IL-1β (C), IL-6 (D), and anti-inflammatory cytokine IL-10 (E) production in Caco-2 cells, respectively. GMP modulated LPS-induced TNF- α (F), IFN-γ (G), IL-1β (H), IL-6 (I), and IL-10 (L) in human intestinal Caco-2 cells. Data represent the mean ± s.d. of six determinations performed in triplicate. All the data sets have been analyzed by One-way ANOVA followed by Tukey's post-hoc test. Different lowercase letters indicate a significant difference ($p < 0.05$) between different concentrations. C: control,

when tested in the mixture, clearly ameliorating the anti-inflammatory effects (Fig. 6). As already confirmed in previous studies, GMP showed a positive effect on the gut microbiota of PKU patients, proving a possible prebiotic role on specific taxa and promoting bacterial diversity and systemic health.

In conclusion, our results provide new preclinical insights regarding the mechanism of action and safety of L-free AAs and GMP at intestinal cells, thus, supporting GMP as a valid and health-promoting product for the dietary management of phenylketonuria (PKU) patients. The main limitation of the work is the evaluation of the oxidative stress in the plasma of PKU patients following the free L-AAs and GMP diet, respectively. It would important as next step to measure the ability of GMP to rescue the oxidative stress in the PKU patient's plasma compared to the PKU patients that consumed free L-AAs diet.

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## CRediT authorship contribution statement

**Carmen Lammi:** Conceptualization, Formal analysis, Supervision, Writing – original draft, Writing – review & editing. **Carlotta Bollati:** Investigation, Data curation, Writing – original draft. **Laura Fiori:** Investigation. **Jianqiang Li:** Investigation. **Melissa Fanzaga:** Investigation. **Lorenza d'Adduzio:** Investigation, Data curation. **Martina Tosi:** Investigation. **Alberto Burlina:** Writing – review & editing. **Gianvincenzo Zuccotti:** Writing – review & editing. **Elvira Verduci:** Supervision, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113258.

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