



Research Article

Bovine whey peptides transit the intestinal barrier to reduce oxidative stress in muscle cells



Alberto R. Corrochano^{a,b}, Anita Ferraretto^c, Elena Arranz^a, Milda Stuknytė^d, Michela Bottani^c, Paula M. O'Connor^a, Phil M. Kelly^a, Ivano De Noni^d, Vitaly Buckin^b, Linda Giblin^{a,*}

^a Teagasc Food Research Centre, Moorepark, Fermoy, Co Cork, Ireland

^b School of Chemistry & Chemical Biology, University College Dublin, Dublin 4, Ireland

^c Dipartimento di Scienze Biomediche per la Salute, Università degli Studi di Milano, LITA, Segrate, Italy

^d Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente, Università degli Studi di Milano, Milan, Italy

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ABSTRACT

Health benefits are routinely attributed to whey proteins, their hydrolysates and peptides based on *in vitro* chemical and cellular assays. The objective of this study was to track the fate of whey proteins through the upper gastrointestinal tract, their uptake across the intestinal barrier and then assess the physiological impact to downstream target cells. Simulated gastrointestinal digestion (SGID) released a selection of whey peptides some of which were transported across a Caco-2/HT-29 intestinal barrier, inhibited free radical formation in muscle and liver cells. In addition, SGID of β -lactoglobulin resulted in the highest concentration of free amino acids (176 nM) arriving on the basolateral side of the co-culture with notable levels of branched chain and sulphur-containing amino acids. *In vitro* results indicate that consumption of whey proteins will deliver bioactive peptides to target cells.

1. Introduction

Bovine whey proteins are considered high quality proteins. They are a rich source of branched chain amino acids (BCAA), well balanced, have encrypted bioactive peptides and well documented health benefits to muscle, immune and redox systems (Patel, 2015). As such, the total whey world trade volume in 2015 was more than 1.7 million tonnes with the sports nutrition sector of particular importance (IDF, 2016). Bovine whey proteins are β -lactoglobulin (β -LG, 50–60%), α -lactalbumin (α -LA, 15–25%), bovine serum albumin (BSA, 6%), lactoferrin (LF, < 3%) and several immunoglobulins (< 10%).

BCAA make up 26% of the amino acid (AA) content of whey proteins and function to promote muscle protein synthesis via stimulation of the rapamycin (mTOR) pathway. In a recent study, 8 elderly men who consumed 1.9 g native whey protein/kg lean body weight for 10 days, had significantly higher levels of myosin and mitochondrial muscle protein synthesis than baseline, whereas consumption of casein had no effect. This benefit of whey was attributed to the high content of BCAA (1159 μ M) in their plasma (Walrand et al., 2016). Essential amino acids (EAA) function to reduce muscle inflammation after strenuous exercise. Purpura et al. (2014) observed that consumption of 48 g of commercial whey protein isolate (WPI) resulted in 229.5 nmol

EAA/mL plasma in 10 resistance-trained men 67 min later. Bioactive peptides derived from whey proteins, if bioavailable, may also play a role in muscle health as suggested by *in vitro* data using whey hydrolysates directly on the C2C12 muscle cell line, for instance, activating the mTOR complex I (Roeseler et al., 2017). However, the effect of whey on muscle is not without controversy with some studies showing limited or no effects on muscle after whey consumption or compared with casein supplementation (Agin et al., 2001; Tipton et al., 2004).

Whey proteins are rich in antioxidant AA including the glutathione precursor Cys (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015). The once-off consumption of 3 g dairy product (2.25 g milk protein concentrate plus 0.75 g whey hydrolysates) per kg body weight significantly increased the antioxidant capacity of plasma in 8 healthy women compared to baseline, albeit no control diet was included (Power-Grant et al., 2016). However, consumption of whey had no effect on antioxidant biomarkers in plasma in other studies (Kim, Paik, Yoon, & Park, 2013; Middleton, Jelen, & Bell, 2004). Several individual whey peptides such as β -LG f(19–29) WYSLAMAASDI, β -LG f(42–46) YVEEL, β -LG f(145–149) MHIRL, α -LA f(101–104) INYW and α -LA f(115–118) LDQW have demonstrated antioxidant activity using *in vitro* chemical assays (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; Sadat et al., 2011).

* Corresponding author.

E-mail address: linda.giblin@teagasc.ie (L. Giblin).

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The ability of whey proteins to reduce the pro-inflammatory cytokines, IL-1 β and IL-6, was proposed as the mechanism by which a 16 day diet containing 20% whey protein protected D-galactosamine-treated rats against hepatotoxicity (Kume, Okazaki, & Sasaki, 2006). A 28 day diet containing 10% WPI also protected stressed rats against liver damage by decreasing lipid peroxidation and increasing plasma levels of the antioxidant tripeptide glutathione (Ashoush, El-Batawy, & El-Shourbagy, 2013).

However, little is known about which whey peptides survive the harsh conditions of the gastrointestinal tract and are absorbed, via passive or active transport, across the intestinal barrier to reach target organs. Recently, whey protein degradation during upper gut transit was tracked in human jejunal effluents and using *in vitro* simulated gastrointestinal digestion (SGID) protocols (Sanchón et al., 2018). SGID is a suitable model to predict whey protein digestion as demonstrated by a correlation coefficient of 0.74 for β -LG and α -LA (Sanchón et al., 2018).

Several studies have employed differentiated Caco-2 monolayers to assess the intestinal absorption of individual milk-derived peptides and AA (Goulart et al., 2014; Picariello et al., 2013). For example, Vermeirssen et al. (2002) demonstrated the transport of anti-hypertensive peptide from β -LG, lactokinin f(142–148) ALMPHIR, across Caco-2 clone Bbe monolayers cultured in Ussing chambers.

The objective of this study was to investigate the bioavailability of SGID commercial whey products and individual whey proteins across Caco-2/HT-29 co-culture to mimic upper gut transit of whey and transport across the intestinal barrier. By secreting mucus, the adenocarcinoma HT-29 improves the Caco-2 model. Using Peptide Ranker score, known bioavailability, presence of known antioxidant AA or presence of BCAA, 6 bioavailable peptides were selected and assayed for their ability to promote antioxidant status in muscle and liver cells and modulate cytokine markers in immune cells.

2. Materials and methods

2.1. Materials

Commercial bovine WPI (Isolac, 91.4% protein content) was purchased from Carbery Food Ingredients (Ireland). β -LG (92.1% β -LG content) and α -LA (93% α -LA content) were obtained from Davisco Foods International (USA). LF (Bioferrin 2000, 95% of LF and 0.02% of iron) was gifted by Glanbia Nutritionals (USA). BSA (98% protein content) was purchased from Sigma-Aldrich (Ireland). Milk-protein-based sport product (MPSPPO, 47% protein content: WPI, whey protein concentrate, calcium caseinate, milk protein concentrate) was purchased from a local retailer. Human TNF- α and IL-1 β /IL-1F2 DuoSet ELISA kits and DuoSet Ancillary Reagent Kit 2 were from R&D Systems (UK). All other reagents were purchased from Sigma-Aldrich.

2.2. SGID

SGID was performed as described by Minekus et al. (2014). Whey protein samples (WPI, β -LG, α -LA, BSA and LF) (1 g powder, which contained approximately 0.94 g protein) were reconstituted in 5 mL of Milli-Q H₂O. MPSPPO was reconstituted at 1.5 g powder/5 mL H₂O (0.71 g protein) following manufacturer's recommendations. As they were liquid formulations, no oral phase was performed. Gastric phase was performed for 2 h using porcine pepsin (2000 U/mL). Intestinal phase was performed for 2 h using pancreatin (100 U/mL) and bile extract (10 mM). To stop the intestinal phase, protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (1 mM) was added. Samples were snap frozen in liquid nitrogen and stored at -80°C . Prior to cell exposures, the osmolarity of SGID proteins was measured by Osmometer Basic (Löser Messtechnik, Germany) and corrected to 300 mOsm/kg H₂O (physiological) using distilled H₂O.

2.3. Cell lines

Cells were grown in 75 cm² tissue culture flasks with the correspondent culture medium and kept in a humidified incubator with a 5% CO₂ air atmosphere at 37 $^{\circ}\text{C}$. The intestinal cell lines, Caco-2 (BSTCL87) and HT-29 (BSTCL132), were purchased from Istituto Zooprofilattico Sperimentale di Brescia (Italy). Routinely, Caco-2 and HT-29 cells were cultured separately in Minimum Essential Medium Eagle (MEM) and Roswell Park Memorial Institute-1640 (RPMI-1640) medium respectively, supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, antibiotics (100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin) and 1 mM sodium pyruvate (only for MEM). Murine myoblasts C2C12 (ATCCCL-1772), human hepatocytes HepG2 (ATCCCHB-8065) and human monocytes THP-1 (ATCCCTIB-202) were purchased from American Type Culture Collection (USA). C2C12 cells were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin. Hepatocytes were cultured in MEM supplemented with 10% FBS, 2 mM L-glutamine, 1% non-EAA, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin. Monocytes were cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin). Passage numbers were between 25 and 40 (Caco-2), 10–25 (HT-29), 6–9 (C2C12), 20–25 (HepG2) and 15–20 (THP-1).

2.4. Co-cultures

70% of Caco-2 and 30% of HT-29 cells were seeded together in RPMI-1640 at a density of 4×10^4 cells/cm, as previously described by Ferraretto et al. (2018). Cell confluence was achieved 4 days after seeding. After 6 days post confluence, transport studies were performed. After 6 days post confluence, co-culture cells showed the presence of (1) microvilli with active enzymes, (2) mucus and (3) barrier properties characterized by a permeability value similar to the human small intestine *in vivo* (Ferraretto et al., 2018).

2.5. Cytotoxicity

Co-cultures growing in 24 well plates were incubated for 2 h with SGID proteins prepared in complete RPMI-1640. Cells were washed with PBS and MTT assays performed using 510 μL thiazolyl blue tetrazolium bromide solution (98 $\mu\text{g}/\text{mL}$) in RPMI-1640 for 4 h, followed by 400 μL DMSO. Absorbance was expressed as relative % of untreated co-culture.

2.6. Co-culture transport studies

Co-cultures were grown in Transwell[®] Millicell[®] 24 insert plates (1.0 μm) assembled to a Millicell[®] 24 well receiver tray (EMD Millipore, USA) and maintained in complete RPMI-1460 medium. Transepithelial electrical resistance (TEER) was measured at 37 $^{\circ}\text{C}$ on 0, 3 and 6 days post-confluence using a Millicell[®]-ERS voltohmmeter (EMD Millipore, USA).

On treatment days, TEER values were recorded, and then co-cultures were gently washed 3 times with Hank's Balanced Salt Solution (HBSS) and incubated for 30 min in HBSS at 37 $^{\circ}\text{C}$. After the acclimatisation period, 175 μg SGID proteins in 400 μL HBSS were added to the apical side of the inserts (0.7 cm² surface area, equates to 250 $\mu\text{g}/\text{cm}^2$) and 800 μL HBSS were added to the basolateral compartments. TEER values were monitored immediately and then again, at 1 h and 2 h. After the 2 h treatment, apical and basolateral solutions were collected and stored at -40°C prior to analysis.

The paracellular permeability was determined using the fluorescent probe lucifer yellow as previously described by Ferraretto et al. (2018). Co-cultures were treated at 6 days post confluence with 175 μg SGID proteins together with 100 μM lucifer yellow in HBSS. After 2 h, apical and basolateral solutions were collected and the fluorescence was

measured at excitation of 398 nm and emission of 518 nm using a fluorescence spectrometer (Perkin Elmer, UK). The apparent permeability coefficient (P_{app}) was calculated following the equation:

$$P_{app}(\text{cm/s}) = [1/(S \cdot C_0)] * dQ/dt$$

where S is the surface area of the insert (0.7 cm^2), C_0 is initial concentration of lucifer yellow added in the apical side and dQ/dt is the amount of lucifer yellow detected in the basolateral compartment as a function of time ($\mu\text{mol/s}$) (Ferraretto et al., 2018).

Co-cultures incubated only with lucifer yellow were included as controls. The transport of lucifer yellow through inserts without co-culture was determined at $4.3 \times 10^{-6} \text{ cm/s}$.

2.7. Amino acid determination

The protocol described by McDermott et al. (2016) was followed to determine the free AA in apical and basolateral compartments using Jeol JLC-500/V AA analyzer (Jeol, Welwyn Garden City, UK) fitted with a Jeol Na+ high performance cation exchange column.

2.8. Peptide identification

Peptides in the apical and basolateral solutions were identified by UPLC-HR-MS using an Acquity UPLC module (Waters, USA) fitted to a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, USA). The samples were eluted on an Aeris PEPTIDE XB-C18 column ($150 \times 2.1 \text{ mm}$, $1.7 \mu\text{m}$, 100 \AA) with a SecurityGuard ULTRA cartridge (Phenomenex, USA) following the analytical conditions previously described (Corrochano et al., 2018). Peptide sequences were identified with the Proteome Discoverer 1.4 software (Thermo Scientific, USA) using the *Bos taurus* database (UniProt taxon ID 9913) as reported in Corrochano et al. (2018). Briefly, settings were as follows: mass accuracy window for precursor ions, 5 ppm; mass accuracy window for fragment ions, 0.02 Da; no fixed modifications; variable modifications: phosphorylation of Ser and Thr, deamidation of Asn, Gln and Arg, oxidation of Met and cyclisation of an N-terminal Gln to pyroglutamic acid. Peptides were searched using the Sequest engine (Eng, McCormack, & Yates, 1994), which is included into the PD 1.4 software and allows the identification of peptides as short as two amino acids (M. Scigelova, personal communication). A strict false discovery rate of peptide identification was set (FDR = 0.01).

The peptides ALPM, GDLE, TKIPA, VEELKPT from β -LG, VGIN from α -LA and AVEGPK from BSA were synthesised and purified following the method previously described by Lafarga, Aluko, Rai, O'Connor, and Hayes (2016) and using the resins: H-Met-HMPB-ChemMatrix, H-Ala-HMPB-ChemMatrix, H-Asn-HMPB-ChemMatrix, H-Glu-HMPB-ChemMatrix, H-Thr-HMPB-ChemMatrix and H-Lys-HMPB-ChemMatrix (PCAS Biomatrix Inc., Canada). Each peptide eluted in a single peak.

2.9. Cellular antioxidant activity

Synthetic peptides were tested for cellular antioxidant activity using C2C12 and HepG2 cells. C2C12 or HepG2 were seeded at 8×10^4 cells/well in 96 well plates, in their culture medium, for 24 or 48 h, respectively. Before treatment, cells were washed twice with PBS and treated with 50 μL of each peptide (final concentrations: 2.5 or 5 mM were selected based on previous *in vitro* studies by others (Komatsu et al., 2019; Lacroix, Chen, Kitts, & Li-Chan, 2017; Le Nevé & Daniel, 2011)), reconstituted in HBSS. 2',7'-dichlorofluorescein di-acetate (DCFH-DA, 50 μL (25 μM final)) was added for 1 h. Cells were washed twice with PBS, and 100 μL 2,2'-azobis(2-methylpropionamide) dihydrochloride (ABAP, 600 μM in HBSS) was added. The plate was immediately placed in a Synergy HT BioTek micro plate reader (USA) at 37 °C. Fluorescence was registered every minute for 1 h with excitation at 485 nm and emission at 535 nm. Negative control (cells treated only with HBSS and DCFH-DA) and positive control for cellular oxidation

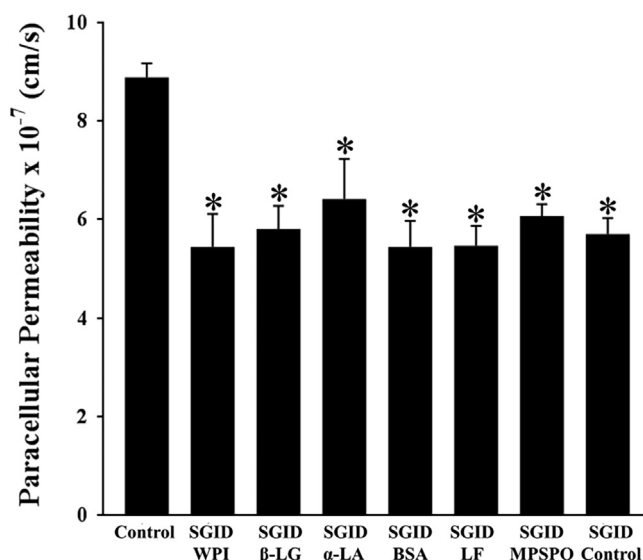


Fig. 1. Caco-2/HT-29 (70:30) paracellular permeability after 2 h treatment with 175 μg simulated gastrointestinal digested (SGID) whey samples: WPI, β -LG, α -LA, BSA, LF, MPSPO, SGID control (gastrointestinal fluids with gut enzymes, bile salts and electrolytes). Caco-2/HT-29 were seeded together at a density of 4×10^4 cells/ cm^2 . Paracellular permeability was measured by lucifer yellow quantification in the basolateral compartment as a % of initial apical concentration (100 μM) in the presence of SGID samples. * $p < 0.05$ indicates significant difference to untreated cells (Control).

(cells incubated only with ABAP) were included. *N*-acetylcysteine (NAC) was used at 2.5 or 5 mM (final concentrations) as the positive control for antioxidant protection. The assay was performed in triplicate on two different days.

2.10. Immunomodulatory activity

THP-1 cells were seeded at 5×10^5 cells/well in 24 well plates. Cells were maintained for 48 h in complete culture medium supplemented with 100 ng/mL of phorbol 12-myristate 13-acetate (PMA) to induce macrophage differentiation. Fresh medium without PMA was added, and cells were grown for 24 h. Macrophages were then washed with PBS and stimulated with 0.05 $\mu\text{g}/\text{mL}$ lipopolysaccharide (LPS) in the presence of peptides (1 μM –5 mM) for 24 h in medium without FBS. After treatment, supernatants were collected and frozen at $-80 \text{ }^\circ\text{C}$. Cytokines TNF- α and IL-1 β were quantified in supernatants by ELISA.

2.11. Peptide cytotoxicity

C2C12 or HepG2 cells were seeded at 8×10^4 cells/well in 96 well plates and allowed to reach confluence for 24 or 48 h, respectively. Cells were washed twice with PBS and treated with peptides (2.5 or 5.0 mM) reconstituted in HBSS for 1 h. Control cells were incubated only with HBSS. After treatment, cells were washed once with PBS and incubated with 50 μL MTT (0.5 mg/mL) in complete medium. For THP-1, cells were differentiated in 24 well plates and then exposed to peptides (1 μM –5 mM) for 24 h. Supernatants were removed and 500 μL MTT solution (0.5 mg/mL) were added. After 3 h, MTT solution was removed, 50 μL (or 500 μL for THP-1 cells) of 1:1 DMSO:ethanol were added and absorbance read at 570 nm in a Synergy HT microplate reader. Cell viability was calculated as a percentage of control.

2.12. Statistical analysis

Co-culture experiments were performed in duplicate on 3 different days. Cellular antioxidant assays were performed in triplicate on 2 different days. Cellular exposures to determine pro-inflammatory

Table 1Free amino acids (nM) in apical and basolateral compartments after 2 h of Caco-2/HT-29 co-culture treatment with 175 µg gastrointestinal digested (SGID) proteins.¹

Amino acid (nM)		SGID WPI	SGID β-LG	SGID α-LA	SGID BSA	SGID LF	SGID MPSP0
Ala	Apical	46.4	46.9	29.6	40.6	47.9	34.8
	Basolateral	21.6	23.8	18.4	21.1	18.9	16.1
Arg	Apical	27.8	28.5	18.8	46.8	60.0	34.8
	Basolateral	23.6	22.5	21.6	23.7	20.9	20.1
Asp	Apical	6.8	6.5	8.4	3.9	6.7	5.9
	Basolateral	0.8	1.9	1.3	1.3	1.2	1.3
Cysteic acid	Apical	9.2	3.3	3.6	3.2	6.1	12.9
	Basolateral	2.6	2.7	2.7	2.8	2.9	1.6
Cys	Apical	19.1	14.8	18.2	14.8	16.8	14.0
	Basolateral	10.3	10.2	6.9	9.4	9.2	9.9
Glu	Apical	29.3	22.1	37.9	24.0	19.6	25.4
	Basolateral	4.2	5.2	5.0	4.8	4.2	4.0
Gly	Apical	11.5	9.4	18.7	9.8	18.9	13.3
	Basolateral	4.5	4.2	4.9	4.6	5.2	4.3
His	Apical	24.0	23.9	21.7	30.0	28.7	25.8
	Basolateral	8.2	9.1	7.7	8.2	7.6	7.3
Ile	Apical	36.3	31.4	35.7	14.9	20.7	24.8
	Basolateral	8.9	9.7	9.6	8.0	7.3	7.8
Leu	Apical	92.6	100.0	72.9	82.9	79.1	78.8
	Basolateral	11.7	17.9	12.3	13.7	11.5	11.1
Lys	Apical	83.3	79.4	88.5	69.8	70.5	73.4
	Basolateral	7.8	11.5	8.9	9.1	7.5	7.4
Met	Apical	16.0	15.1	10.6	5.1	8.4	13.0
	Basolateral	2.1	2.9	1.9	1.6	1.5	1.7
Phe	Apical	30.2	26.7	36.7	56.9	47.2	36.5
	Basolateral	3.8	5.1	5.0	5.7	4.7	4.4
Ser	Apical	18.5	17.8	13.1	19.5	22.3	18.9
	Basolateral	6.0	6.4	6.5	6.7	6.0	5.2
Tau	Apical	8.5	7.5	8.9	7.2	8.0	8.2
	Basolateral	10.8	9.2	8.9	9.4	9.0	5.0
Thr	Apical	39.8	26.1	40.2	31.3	36.8	30.0
	Basolateral	11.4	10.6	11.1	11.8	9.2	9.2
Trp	Apical	45.5	35.6	79.0	23.2	53.6	34.8
	Basolateral	6.0	5.7	6.5	4.5	4.6	5.5
Tyr	Apical	27.5	29.5	31.6	41.5	33.0	32.6
	Basolateral	4.9	5.7	5.4	5.6	5.1	5.6
Val	Apical	48.7	37.1	40.3	41.5	45.8	38.1
	Basolateral	9.8	12.2	6.6	9.8	9.3	9.5
Total	Apical	621.1	561.7	614.4	566.9	630.0	556.0
	Basolateral	159.1	176.5	151.2	161.9	145.8	137.1

¹ WPI = Whey protein isolate.

β-LG = β-Lactoglobulin.

α-LA = α-Lactalbumin.

BSA = Bovine serum albumin.

LF = Lactoferrin.

MPSP0 = Milk protein sport product.

biomarkers were performed in duplicate on 2 different days. One-way ANOVA followed by Bonferroni's Multiple Comparison post-hoc test was employed to compare results using the PASW Statistics 18 software. P-value < 0.05 indicated statistical significance. Results were expressed as means ± standard deviations.

3. Results

3.1. Co-culture integrity and cytotoxicity of SGID proteins

Whey products (WPI, MPSP0) and individual whey proteins (β-LG, α-LA, BSA, LF) were subjected to static upper SGID in duplicate. As SGID samples can be cytotoxic to cells, 3 concentrations were initially tested (10.5, 50.0, 250.0 µg protein/cm²) by MTT assay. These initial concentrations were selected based on the total surface area of the small intestine (200 m²) together with (a) the recommended intake of MPSP0 (21 g protein/day) which equates to 10 µg protein/cm², (b) daily high protein intake in a western diet (100 g/day) (50 µg protein/cm²) or (c) a 5 fold increase to compensate for *in vitro* lack of microclimate (250.0 µg protein/cm²). None of the tested SGID sample concentrations (10.5, 50.0, 250.0 µg protein/cm²) were cytotoxic to Caco-2/HT-29co-

culture (Fig. S1A) so the highest concentration was selected (250.0 µg protein/cm²) to proceed to transport studies.

After 6 days post confluence, co-cultures showed absorptive and secretive enteric phenotypes (Ferraretto et al., 2018) allowing commencement of transport studies with SGID samples. Co-culture integrity was monitored during transport studies at 0, 1 and 2 h. TEER values were not altered due to sample exposure (190–250 Ω/cm²) and did not significantly change over time (Fig. S1B).

To evaluate if SGID proteins (250 µg protein/cm²) interfered with intestinal epithelium permeability, the transport of the fluorescent probe lucifer yellow was assessed (Fig. 1). The permeability of the lucifer yellow through the untreated co-culture was 8.9×10^{-7} cm/s. All SGID protein samples, including SGID control (gastrointestinal fluids with gut enzymes and bile extract), reduced intestinal permeability from 5.1×10^{-7} to 6.1×10^{-7} cm/s.

3.2. Free amino acids transport

Free AA were quantified in the apical and basolateral compartments after incubating Caco-2/HT-29 co-cultures with SGID proteins for 2 h (Table 1). The apical sample from LF treated co-cultures had the highest

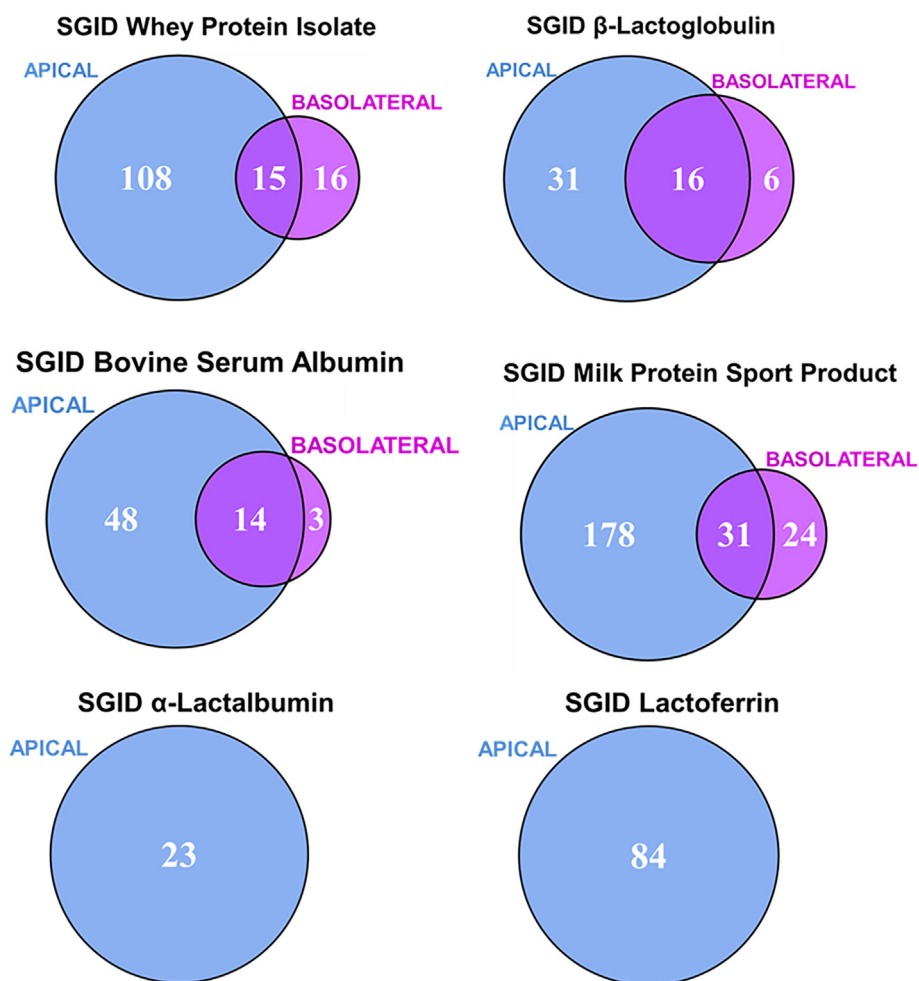


Fig. 2. Number of peptides found in SGID samples in the apical (blue circle) and basolateral (purple circle) compartments of Caco-2/HT-29 co-cultures treated for 2 h with 175 μ g SGID whey protein isolate, β -lactoglobulin, bovine serum albumin, milk protein sport product, α -lactalbumin and lactoferrin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentration of free AA (630 nM), followed by the apical samples from WPI (621.1 nM) and α -LA (614.4 nM). Leu (72.9–100 nM) and Lys (69.8–88.5 nM) were the most abundant AA in the apical compartments. Analysis of the basolateral compartments revealed that the amount of free AA was highest for β -LG (176.5 nM), BSA (161.9 nM) and WPI (159.1 nM) treated co-cultures. Most abundant AA in the basolateral solutions of co-cultures treated with whey proteins were Arg (20.1–23.7 nM), Ala (16.1–23.8 nM) and Leu (11.1–18.0 nM). Co-cultures treated with β -LG had the highest concentration of EAA (84.7 nM), BCAA (39.9 nM) and sulphur-containing AA (15.9 nM) in the basolateral solution (Table 1). The antioxidant AA, Trp, was the most abundant in the basolateral sample collected from co-cultures treated with α -LA (6.5 nM).

3.3. Peptide transport

Peptide sequences identified by UPLC-HR-MS in the apical and basolateral samples from co-cultures treated with SGID whey samples are listed in Tables S1–S6.

The apical solution of WPI-treated co-cultures contained 123 peptides of which 15 were also detected in the basolateral solution with a further 16 unique to basolateral (Fig. 2). The apical solution of β -LG showed 47 peptides with 16 common to basolateral and 6 peptides exclusive to the basolateral compartment. The apical solution of BSA-treated cells yielded 62 peptides of which 14 were common to basolateral and a further 3 unique to the basolateral solution. The MPSP

showed 209 peptides, including casein peptides, in the apical solution with 31 common to basolateral and 24 additional peptides in the basolateral compartment. Whereas 23 and 84 peptides were identified in the apical side of α -LA- and LF-treated co-cultures respectively, no peptides were identified in the corresponding basolateral solutions at the 250 μ g protein/cm² concentration tested.

From the peptides identified in basolateral solutions, 6 peptides were synthesized for bioactivity testing based on (a) a high Peptide Ranker score (ALPM (derived from β -LG) = 0.82, Table S1), (b) known bioavailability (GDLE (β -LG) and VEELKPT (β -LG)), (c) presence of known antioxidant AA (ALPM, AVEGPK (BSA), GDLE and TKIPA (β -LG) or (d) presence of BCAA (VEELKPT, VGIN (α -LA)).

3.4. Cellular antioxidant protection of milk peptides

The antioxidant benefit to muscle cells of the synthesised peptides were investigated by measuring the peptide ability to inhibit intracellular peroxy radical formation in a mouse muscle cell line C2C12 (Fig. 3A). Results were expressed as % of cells treated only with the radical producer ABAP, considered as 100% cellular oxidative stress. Peptide VEELKPT at both concentrations tested (2.5, 5 mM) significantly reduced C2C12 viability (72.4% and 93.8%, respectively) and was therefore omitted. Peptides ALPM, GDLE, VGIN and AVEGPK (5 mM) reduced cellular oxidation in ABAP-treated muscle cells, 34.4%–53%, compared to the free radical control and similar to the effect obtained with the antioxidant molecule NAC (31.8%). For HepG2

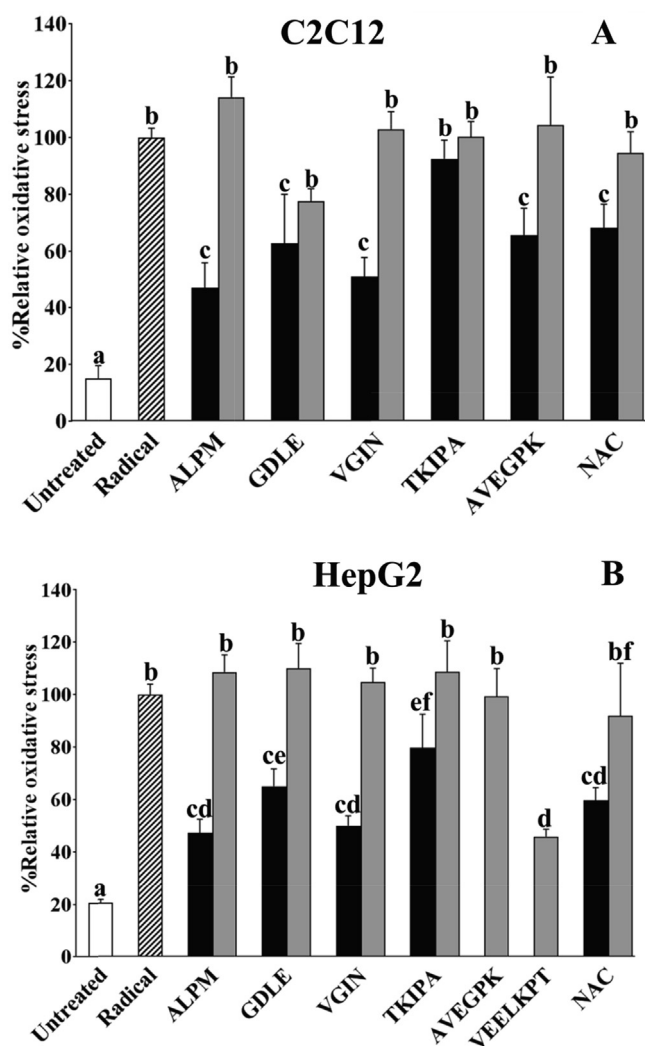


Fig. 3. Relative oxidative stress in (A) muscle (C2C12) and (B) liver (HepG2) cells after 1 h exposure to 5 (black bars) or 2.5 mM (grey bars) synthesised peptides derived from whey: ALPM (β -LG), GDLE (β -LG), TKIPA (β -LG), VEELKPT (β -LG) VGIN (α -LAC) and AVEGPK (BSA) or N-acetylcysteine (NAC) (positive control). C2C12 and HepG2 cells were seeded at a density of 8×10^4 cells/well for 24 h or 48 h, respectively, before peptide exposure. VEELKPT was cytotoxic to C2C12 cells at 2.5 and 5 mM. VEELKPT and AVEGPK at 5 mM were cytotoxic to HepG2. Results are expressed as % relative to cells treated only with the peroxy radical producer ABTS, labelled “radical”. “Untreated” corresponds to cells incubated only with Hank’s Balanced Salt Solution. * $p < 0.05$ indicates significant difference to radical control.

liver cells, peptides VEELKPT (5 mM) and AVEGPK (5 mM) were omitted as they significantly reduced cell viability by 94.1% and 92%, respectively. The levels of oxidation significantly decreased between 35 and 52.6% in stressed ABAP-treated HepG2 cells with the treatment of ALPM, GDLE or VGIN (5 mM). Peptide VEELKPT (2.5 mM) was the most potent in decreasing cellular oxidation (51.1%) although it also reduced cell viability by a notable 30%.

3.5. Effect of milk peptides on the release of pro-inflammatory cytokines

THP-1 monocytes were initially differentiated into macrophages and then activated with LPS for 24 h prior to peptide exposure. Secreted levels of the pro-inflammatory cytokines, TNF- α and IL-1 β , from THP-1 macrophages exposed to peptides (1 μ M–5 mM) are depicted in Fig. 4. Peptides ALPM, GDLE, VEELKPT, VGIN and AVEGPK (5 mM) significantly increased the levels of IL-1 β (131.9–245.5%) in activated

macrophages compared to the LPS control ($p < 0.05$). Secretion of TNF- α was not altered after peptide treatment at any of the concentrations tested ($p > 0.05$). Peptides were not cytotoxic to macrophages after 24 h incubation (data not represented).

4. Discussion

Our study identified whey peptides, post SGID, that crossed Caco-2/HT29 barriers. Of 6 bioavailable peptides selected, 4 inhibited free radicals in muscles and liver cells, 2 reduced cellular viability of liver cells and 5 increased secretion of IL-1 β from stimulated macrophages. In addition, SGID of β -LG resulted in the highest concentration of free AA (176 nM) arriving on the basolateral face of the intestinal barrier with notable levels of BCAA and sulphur-containing AA.

Picariello et al. (2013) identified peptides primarily derived from two regions within β -LG, (40–60AA and 125–135AA) and 3 α -LA peptides, within regions 56–69AA and 114–121AA, in the basolateral compartment of Caco-2 cells treated with SGID WPI. In agreement we also identified β -LG peptides from these regions (VEELKPTPE, GDLE and EVDDE) in the basolateral solution after co-culture treatment with SGID WPI and β -LG. α -LA f(99–102) VGIN peptide was also transported across our co-culture after exposure to WPI and MPSP0. The absence of α -LA and LF peptides in the basolateral solutions agrees with the limited number of LF peptides identified in human jejunum by Boutrou et al. (2013) and the noteworthy rapid digestion of α -LA into free AA (Pantako & Amiot, 2001). The large number of casein-derived compared to whey-derived peptides in MPSP0 agrees with reports that there are fewer peptides released from whey proteins than from casein after gut transit (Boutrou et al., 2013). Surprisingly, several casein-derived peptides were also identified in both the apical and basolateral compartments of Caco-2/HT-29 barriers exposed to WPI, which suggest casein peptide leakage from the casein micelle during thermal processing. Five peptides from β -CN region 133–142AA were found in the apical solution of co-cultures treated with MPSP0 of which only β -CN f(134–139) HLPLPL was transported across the barrier. In agreement, Wang, Wang, and Li (2016) observed HLPLPL in the basolateral side of Caco-2 monolayers treated with SGID casein hydrolysates (10 mg/mL) for 2 h. Similar to our results, peptides from β -CN 81–92AA were also abundant in the basolateral compartment (Wang et al., 2016). Agreement of bioavailable peptides in our study with others (Picariello et al., 2013; Wang et al., 2016), together with absorptive and secretive enteric phenotype data (Ferraretto et al., 2018) supports our view that the co-culture model compares well with the established 21 day differentiated Caco-2 model.

Previously, β -LG peptides f(15–18) VAGT, f(24–26) MAA and f(71–74) IIAE inhibited peroxy radicals, using the oxygen reactive absorbance capacity (ORAC) assay with values between 0.33 and 1.79 μ mol trolox equivalents (TE)/mmol peptide (O’Keeffe, Conesa, & FitzGerald, 2017). Of these, our study revealed that the peptides VAGT and IIAE were able to pass through the intestinal co-culture.

Previously, β -LG peptides f(19–29) WYSLAMAASDI, f(145–149) MHIRL, f(42–46) YVEEL and many of their derivatives exhibited antioxidant activity by ORAC (0.306–2.621 μ mol TE/ μ mol peptide) (Hernández-Ledesma et al., 2005). Additionally, other studies have provided evidence that YVEEL or its derivatives survive gut transit *in vivo* and *in vitro*. Our study and that of Picariello et al. (2013) confirmed transport of the related peptide VEELKPT across the intestinal barrier. However, VEELKPT (5 mM) is cytotoxic to HepG2 and C2C12 cells undermining its ability to reduce oxidative stress at 2.5 mM in HepG2 cells. β -LG peptide f(76–81) TKIPAV was identified in the most antioxidant fraction of infant formula post SGID (Hernandez-Ledesma, Quirós, Amigo, & Recio, 2007). Derivatives of TKIPA, peptides TKIPAVFK and KIPAVFKIDAL, were also found in antioxidant fractions of buttermilk, whey proteins and skim milk powder (Bertucci, Liggieri, Colombo, Vairo Cavalli, & Bruno, 2015; Conway, Gauthier, & Pouliot, 2012). We confirmed transport across the intestinal barrier of TKIPA

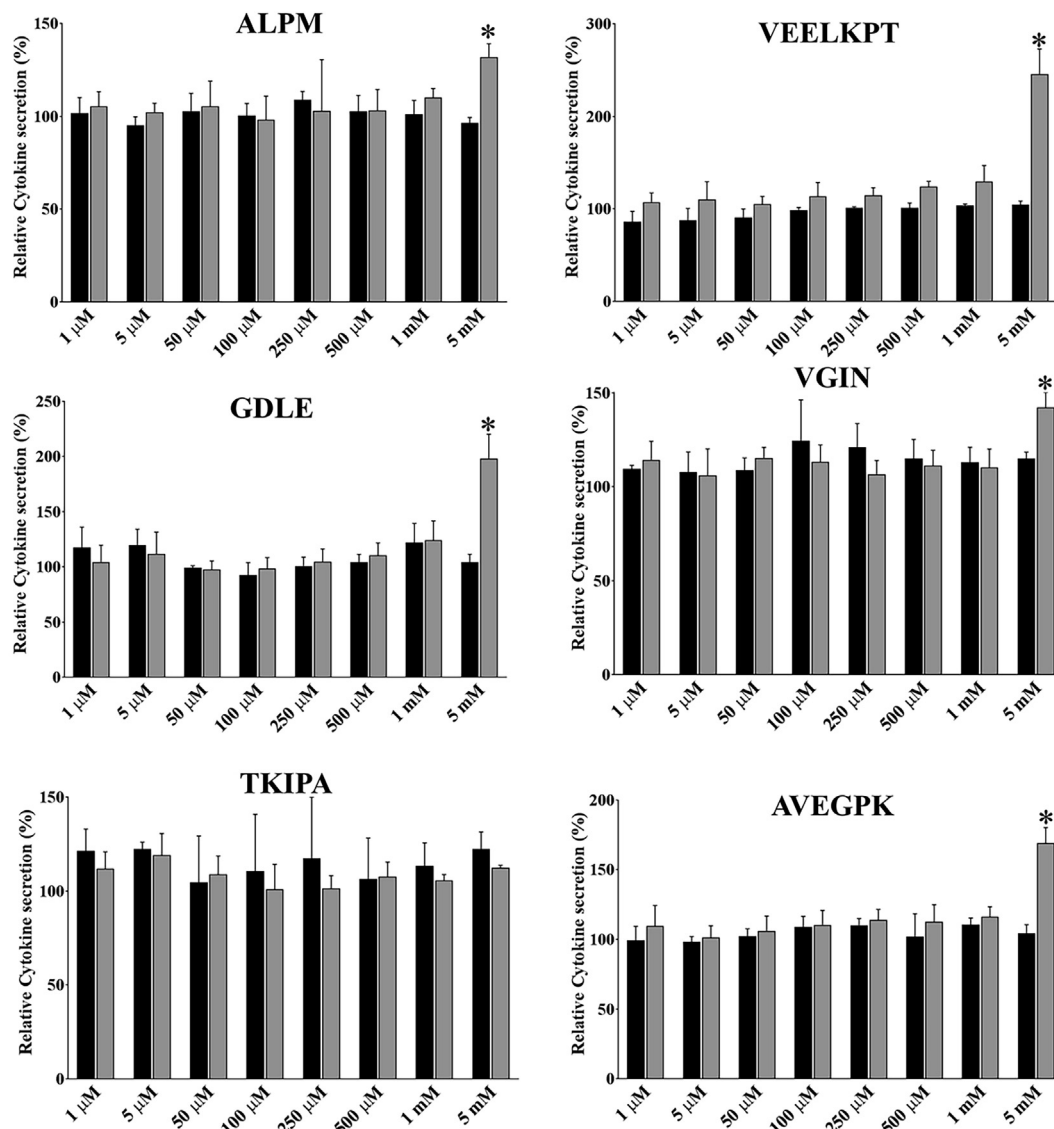


Fig. 4. Relative secretion of TNF- α (black bars) and IL-1 β (grey bars) from LPS-stimulated macrophages after 24 h peptide treatment (1 μ M–5 mM) expressed as % of LPS-activated cells without peptide treatment (100%). Unstressed macrophages (differentiated THP-1 cells, seeded at 5×10^5 cells/well) were considered as negative control with values of $2.6 \pm 0.2\%$ for TNF- α and $3.2 \pm 0.5\%$ for IL-1 β . * $p < 0.05$ indicates significant difference to LPS-stimulated macrophages.

but it did not protect liver and muscle cells against radicals (2.5–5 mM).

Peptide PEGDLEI (β -LG 50–56) was detected in whey hydrolysed by Corolase PP (Mann et al., 2015) which was capable of scavenging the synthetic radical 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid). In our study, related peptide GDLE (5 mM) survived gut transit, was bioavailable, boosted antioxidant cellular response and protected stressed hepatocytes against cellular oxidation. The peptides f (101–104) INYW and f(115–118) LDQW at 2.5 μ M derived from α -LA inhibited ABTS by 100% (Sadat et al., 2011). Although LDQW was not detected in the basolateral chamber of our Caco-2/HT-29 co-culture, VGIN was and exerted an antioxidant protection on both C2C12 and HepG2 cells.

Peptides ALPM and AVEGPK from β -LG have not been previously reported as antioxidants, however, they possess hydrophobic AA at the N or C terminus, a common characteristic of antioxidant peptides (Nielsen, Beverly, Qu, & Dallas, 2017). Both peptides (5 mM) counteracted free radicals in C2C12. ALPM also exerted an antioxidant effect in HepG2 cells. In total, 3 dipeptides were identified in the basolateral compartments post Caco-2/HT-29 exposure to β -LG, BSA and MPSPO of which EL (5 mM) had proven antioxidant activity by inhibiting the synthetic radical 1,1-diphenyl-2-picrylhydrazyl (Suetsuna, Ukeda, &

Ochi, 2000).

Peptides ALPM, GDLE, VEELKPT, VGIN and AVEGPK (5 mM) increased the pro-inflammatory cytokine IL-1 β , but levels of TNF- α remained unchanged. TNF- α also remained unchanged in vascular endothelial cells after direct treatment with 0.5–5.0 mg/mL WPI or hydrolysates ($p > 0.05$) (Da Silva, Bigo, Barbier, & Rudkowska, 2017). However, TNF- α was significantly increased, 10–200 fold, in THP-1 cells after exposure to intact or hydrolysed WPI (2 mg/mL) (Kiewiet et al., 2017). Human intervention studies also provide conflicting results about the role of dairy products in the immune system (Bordoni et al., 2017). Meyer, Elmadfa, Herbacek, and Micksche (2007) reported increased levels of IL-1 β (40%) and TNF- α (63%) on 33 healthy young women receiving 100–200 g yogurt/day compared to baseline. In contrast, 3 h after ingestion of 400 mL reduced fat milk significantly decreased plasma IL-1 β (31%) and TNF- α (27%) in 12 overweight subjects (Nestel et al., 2012).

It is interesting to note that several peptides identified in the apical and basolateral had proven bioactivities other than antioxidant and immunomodulation. Peptides GDLE, ALPM and VGIN were encrypted within the peptides PEGDL, LPMH, KVGIN. These latter peptides were identified in WPI hydrolysates produced by pepsin and were active

against *Listeria ivanovii* at 37.5 mg/mL (Theolier, Hammami, Labelle, Fliss, & Jean, 2013). The peptides TKIPA and ALPM share 3 AA with LPMH and IPA that have proven antihypertensive activity by inhibiting the angiotensin-I-converting enzyme (ACE) *in vitro*. Additionally, in hypertensive rats, IPA (8 mg/kg) reduced the systolic blood pressure by 31 mm Hg after 6 h administration (Mullally, Meisel, & FitzGerald, 1997; Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998). The BSA peptide f(568–573) AVEGPK was found in a bioactive fraction of *Phaseolus vulgaris* post SGID which was capable of 50% ACE inhibition when assayed at 105.6 mg peptide/mL (Tagliazucchi, Martini, Bellesia, & Conte, 2015).

Arg, Ala and Leu were predominant in the basolateral side, at concentrations of 11.1 nM (Leu in SGID MPSP0) to 23.8 nM (Ala in β -LG). Goulart et al. (2014) also reported that Arg and Leu were predominant basolateral AA (21.8% and 4.1% respectively) post Caco-2 treatment with SGID fresh whey (6 μ g/ μ L). The absence of Pro diffusion was notable in this study and agrees with previous data that Pro-containing peptides are resistant to gut enzymatic hydrolysis and epithelial proteases suggesting that peptides surviving the gut are likely to contain Pro (Boutrou et al., 2013). It also agrees with our data, where Pro appeared in 73% of the peptides identified in the basolateral compartment.

β -LG (250 μ g protein/cm²) delivers the most EAA (84.7 nM), BCAA (39.9 nM) and sulphur-containing AA (15.9 nM) to the basolateral. Interestingly, Caco-2/HT-29 exposure to SGID α -LA (250 μ g protein/cm²) resulted in the highest concentration of the antioxidant AA, Trp (6.5 nM). In a human intervention study, a diet supplemented with 141 g of α -LA increased the ratio between Trp and large neutral AA by 43% compared to the control diet containing casein (Markus, Olivier, & de Haan, 2002).

Co-culture treatment with SGID proteins did not alter TEER values, whereas the paracellular permeability of lucifer yellow significantly decreased in the presence of milk peptides compared to untreated cells. This intestinal modulation may arise from the presence of transforming growth factor- β in whey proteins, which is thought to increase the tight junction protein claudin-4 (Hering et al., 2011). The rate and mode of transport (passive V active) of the bioavailable peptides across the intestinal co-culture were not investigated in our study.

5. Conclusion

This study provides evidence that individual whey peptides survive gut transit, are bioavailable across the intestinal barrier and are bioactive on muscle and liver cell lines.

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2019.03.009>.

References

- Abubakar, A., Saito, T., Kitazawa, H., Kawai, Y., & Itoh, T. (1998). Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. *Journal of Dairy Science*, *81*(12), 3131–3138. [https://doi.org/10.3168/jds.S0022-0302\(98\)75878-3](https://doi.org/10.3168/jds.S0022-0302(98)75878-3).
- Agin, D., Gallagher, D., Wang, J., Heymsfield, S. B., Pierson, R. N., Jr, & Kotler, D. P. (2001). Effects of whey protein and resistance exercise on body cell mass, muscle strength, and quality of life in women with HIV. *Aids*, *15*(18), 2431–2440.
- Ashoush, I. S., El-Batawy, O. I., & El-Shourbagy, G. A. (2013). Antioxidant activity and hepatoprotective effect of pomegranate peel and whey powders in rats. *Annals of Agricultural Sciences*, *58*(1), 27–32. <https://doi.org/10.1016/j.aos.2013.01.005>.
- Bertucci, J. I., Liggieri, C. S., Colombo, M. L., Cavalli, S. E. V., & Bruno, M. A. (2015). Application of peptidases from *Maclura pomifera* fruit for the production of active biopeptides from whey protein. *LWT-Food Science and Technology*, *64*(1), 157–163. <https://doi.org/10.1016/j.lwt.2015.05.041>.
- Bordonio, A., Danesi, F., Dardevet, D., Dupont, D., Fernandez, A. S., Gille, D., Nunes Dos Santos, C., Pinto, P., Re, R., Remond, D., Shahar, D. R., & Vergeres, G. (2017). Dairy products and inflammation: a review of the clinical evidence. *Critical Reviews in Food Science and Nutrition*, *57*(12), 2497–2525. <https://doi.org/10.1080/10408398.2014.967385>.
- Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., ... Leonil, J. (2013). Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans—The American Journal of Clinical Nutrition, *97*(6), 1314–1323. <https://doi.org/10.3945/ajcn.112.055202>.
- Conway, V., Gauthier, S. F., & Pouliot, Y. (2012). Antioxidant activities of buttermilk proteins, whey proteins, and their enzymatic hydrolysates. *Journal of Agricultural and Food Chemistry*, *61*(2), 364–372. <https://doi.org/10.1021/jf304309g>.
- Corrochano, A. R., Arranz, E., De Noni, I., Stuknyte, M., Ferraretto, A., Kelly, P. M., ... Giblin, L. (2018). Intestinal health benefits of bovine whey proteins after simulated gastrointestinal digestion. *Journal of Functional Foods*, *49*(October), 526–535. <https://doi.org/10.1016/j.jff.2018.08.043>.
- Da Silva, M. S., Bigo, C., Barbier, O., & Rudkowska, I. (2017). Whey protein hydrolysate and branched-chain amino acids downregulate inflammation-related genes in vascular endothelial cells. *Nutrition Research*, *38*, 43–51. <https://doi.org/10.1016/j.nutres.2017.01.005>.
- Eng, J. K., McCormack, A. L., & Yates, J. R., III (1994). An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *Journal of the American Society for Mass Spectrometry*, *5*(11), 976–989. [https://doi.org/10.1016/1044-0305\(94\)80016-2](https://doi.org/10.1016/1044-0305(94)80016-2).
- Ferraretto, A., Bottani, M., De Luca, P., Cornaghi, L., Arnaboldi, F., Maggioni, M., ... Donetti, E. (2018). Morpho-functional properties of a differentiated Caco2/HT-29 co-culture as an in vitro model of human intestinal epithelium. *Bioscience Reports*, *38*. <https://doi.org/10.1042/BSR20171497>.
- Fox, P. F., Uniacke-Lowe, T., McSweeney, P. L. H., & O'Mahony, J. A. (2015). Milk proteins. In P. F. Fox, T. Uniacke-Lowe, P. L. H. McSweeney, & J. A. O'Mahony (Eds.). *Dairy Chemistry and Biochemistry* (pp. 145–240). New York: Springer International Publishing.
- Goulart, A. J., Bassan, J. C., Barbosa, O. A., Marques, D. P., Silveira, C. B., Santos, A. F., ... Monti, R. (2014). Transport of amino acids from milk whey by Caco-2 cell monolayer after hydrolytic action of gastrointestinal enzymes. *Food Research International*, *63*, 62–70. <https://doi.org/10.1016/j.foodres.2014.01.037>.
- Hering, N. A., Andres, S., Fromm, A., van Tol, E. A., Amasheh, M., Mankertz, J., ... Schulzke, J. D. (2011). Transforming growth factor- β , a whey protein component, strengthens the intestinal barrier by upregulating claudin-4 in HT-29/B6 cells. *The Journal of Nutrition*, *141*(5), 783–789. <https://doi.org/10.3945/jn.110.137588>.
- Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., & Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from α -lactalbumin and β -lactoglobulin. Identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, *53*(3), 588–593. <https://doi.org/10.1021/jf048626m>.
- Hernandez-Ledesma, B., Quiros, A., Amigo, L., & Recio, I. (2007). Identification of bioactive peptides after digestion of human milk and infant formula with pepsin and pancreatin. *International Dairy Journal*, *17*(1), 42–49. <https://doi.org/10.1016/j.idairyj.2005.12.012>.
- IDF (2016). *The World Dairy Situation 2016*. Bulletin of the International Dairy Federation485.
- Kiewiet, M. G., Dekkers, R., Gros, M., van Neerven, R. J., Groeneveld, A., de Vos, P., & Faas, M. M. (2017). Toll-like receptor mediated activation is possibly involved in immunoregulating properties of cow's milk hydrolysates. *PLoS one*, *12*(6), e0178191. <https://doi.org/10.1371/journal.pone.0178191>.
- Kim, J., Paik, H. D., Yoon, Y. C., & Park, E. (2013). Whey protein inhibits iron overload-induced oxidative stress in rats. *Journal of Nutritional Science and Vitaminology*, *59*(3), 198–205.
- Komatsu, Y., Wada, Y., Izumi, H., Shimizu, T., Takeda, Y., Hira, T., & Hara, H. (2019). Casein materials show different digestion patterns using an in vitro gastrointestinal model and different release of glucagon-like peptide-1 by enteroendocrine GLUTag cells. *Food Chemistry*, *277*, 423–431. <https://doi.org/10.1016/j.foodchem.2018.10.123>.
- Kume, H., Okazaki, K., & Sasaki, H. (2006). Hepatoprotective effects of whey protein on D-galactosamine-induced hepatitis and liver fibrosis in rats. *Bioscience, Biotechnology, and Biochemistry*, *70*(5), 1281–1285. <https://doi.org/10.1271/bbb.70.1281>.
- Lacroix, I. M., Chen, X. M., Kitts, D. D., & Li-Chan, E. C. (2017). Investigation into the bioavailability of milk protein-derived peptides with dipeptidyl-peptidase IV inhibitory activity using Caco-2 cell monolayers. *Food & Function*, *8*(2), 701–709. <https://doi.org/10.1039/c6fo01411a>.

- Lafarga, T., Aluko, R. E., Rai, D. K., O'Connor, P., & Hayes, M. (2016). Identification of bioactive peptides from a papain hydrolysate of bovine serum albumin and assessment of an antihypertensive effect in spontaneously hypertensive rats. *Food Research International*, 81, 91–99. <https://doi.org/10.1016/j.foodres.2016.01.007>.
- Le Nevé, B., & Daniel, H. (2011). Selected tetrapeptides lead to a GLP-1 release from the human enteroendocrine cell line NCI-H716. *Regulatory Peptides*, 167(1), 14–20. <https://doi.org/10.1016/j.regpep.2010.10.010>.
- Mann, B., Kumari, A., Kumar, R., Sharma, R., Prajapati, K., Mahboob, S., & Athira, S. (2015). Antioxidant activity of whey protein hydrolysates in milk beverage system. *Journal of Food Science and Technology*, 52(6), 3235–3241. <https://doi.org/10.1007/s13197-014-1361-3>.
- Markus, C. R., Olivier, B., & de Haan, E. H. (2002). Whey protein rich in α -lactalbumin increases the ratio of plasma tryptophan to the sum of the other large neutral amino acids and improves cognitive performance in stress-vulnerable subjects. *The American Journal of Clinical Nutrition*, 75(6), 1051–1056. <https://doi.org/10.1093/ajcn/75.6.1051>.
- McDermott, A., Visentin, G., De Marchi, M., Berry, D. P., Fenelon, M. A., O'Connor, P. M., ... McParland, S. (2016). Prediction of individual milk proteins including free amino acids in bovine milk using mid-infrared spectroscopy and their correlations with milk processing characteristics. *Journal of Dairy Science*, 99(4), 3171–3182. <https://doi.org/10.3168/jds.2015-9747>.
- Meyer, A. L., Elmaddfa, I., Herbacek, I., & Micksche, M. (2007). Probiotic, as well as conventional yogurt, can enhance the stimulated production of proinflammatory cytokines. *Journal of Human Nutrition and Dietetics*, 20(6), 590–598. <https://doi.org/10.1111/j.1365-277X.2007.00807>.
- Middleton, N., Jelen, P., & Bell, G. (2004). Whole blood and mononuclear cell glutathione response to dietary whey protein supplementation in sedentary and trained male human subjects. *International Journal of Food Sciences and Nutrition*, 55(2), 131–141.
- Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., ... Brodtkorb, A. (2014). A standardised static in vitro digestion method suitable for food – An international consensus. *Food & Function*, 5(6), 1113–1124. <https://doi.org/10.1039/C3FO60702J>.
- Mullally, M. M., Meisel, H., & FitzGerald, R. J. (1997). Identification of a novel angiotensin-I-converting enzyme inhibitory peptide corresponding to a tryptic fragment of bovine β -lactoglobulin. *FEBS Letters*, 402(2–3), 99–101. [https://doi.org/10.1016/S0014-5793\(96\)01503-7](https://doi.org/10.1016/S0014-5793(96)01503-7).
- Nestel, P. J., Pally, S., Macintosh, G. L., Greeve, M. A., Middleton, S., Jowett, J., & Meikle, P. J. (2012). Circulating inflammatory and atherogenic biomarkers are not increased following single meals of dairy foods. *European Journal of Clinical Nutrition*, 66(1), 25. <https://doi.org/10.1038/ejcn.2011.134>.
- Nielsen, S. D., Beverly, R. L., Qu, Y., & Dallas, D. C. (2017). Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. *Food Chemistry*, 232, 673–682. <https://doi.org/10.1016/j.foodchem.2017.04.056>.
- O'Keefe, M. B., Conesa, C., & FitzGerald, R. J. (2017). Identification of angiotensin converting enzyme inhibitory and antioxidant peptides in a whey protein concentrate hydrolysate produced at semi-pilot scale. *International Journal of Food Science & Technology*, 52(8), 1751–1759. <https://doi.org/10.1111/ijfs.13448>.
- Pantako, O. T., & Amiot, J. (2001). The effects of α -lactalbumin and whey protein concentrate on α -amino acids, calcium and phosphorus levels in blood and gastrointestinal tract of rats. *Reproduction Nutrition Development*, 41(3), 227–238. <https://doi.org/10.1051/rnd:2001126>.
- Patel, S. (2015). Emerging trends in nutraceutical applications of whey protein and its derivatives. *Journal of Food Science and Technology*, 52(11), 6847–6858. <https://doi.org/10.1007/s13197-015-1894-0>.
- Picariello, G., Iacomino, G., Mamone, G., Ferranti, P., Fierro, O., Gianfrani, C., ... Addeo, F. (2013). Transport across Caco-2 monolayers of peptides arising from in vitro digestion of bovine milk proteins. *Food Chemistry*, 139(1–4), 203–212. <https://doi.org/10.1016/j.foodchem.2013.01.063>.
- Power-Grant, O., McCormack, W. G., Ramia De Cap, M., Amigo-Benavent, M., Fitzgerald, R. J., & Jakeman, P. (2016). Evaluation of the antioxidant capacity of a milk protein matrix in vitro and in vivo in women aged 50–70 years. *International Journal of Food Sciences and Nutrition*, 67(3), 325–334. <https://doi.org/10.3109/09637486.2016.1153607>.
- Purpura, M., Lowery, R. P., Joy, J. M., De Souza, E. O., Kalman, D., Jäger, R., & Wilson, J. (2014). A comparison of blood amino acid concentrations following ingestion of rice and whey protein isolate: A double-blind, crossover study. *Journal of Nutrition and Health Sciences*, 1(3), <https://doi.org/10.15744/2393-9060.1.306>.
- Roeseler, D. A., McGraw, N. J., Butteiger, D. N., Shah, N., Hall-Porter, J., Mukherjee, R., & Krul, E. S. (2017). Muscle protein signaling in C2C12 cells is stimulated to similar degrees by diverse commercial food protein sources and experimental soy protein hydrolysates. *Journal of Agricultural and Food Chemistry*, 65(14), 2956–2964. <https://doi.org/10.1021/acs.jafc.6b05460>.
- Sadat, L., Cakir-Kiefer, C., N'Negue, M. A., Gaillard, J. L., Girardet, J. M., & Miclo, L. (2011). Isolation and identification of antioxidative peptides from bovine α -lactalbumin. *International Dairy Journal*, 21(4), 214–221. <https://doi.org/10.1016/j.idairyj.2010.11.011>.
- Sanchón, J., Fernández-Tomé, S., Miralles, B., Hernández-Ledesma, B., Tomé, D., Gaudichon, C., & Recio, I. (2018). Protein degradation and peptide release from milk proteins in human jejunum. Comparison with in vitro gastrointestinal simulation. *Food Chemistry*, 239, 486–494. <https://doi.org/10.1016/j.foodchem.2017.06.134>.
- Suetsuna, K., Ukedda, H., & Ochi, H. (2000). Isolation and characterization of free radical scavenging activities peptides derived from casein. *The Journal of Nutritional Biochemistry*, 11(3), 128–131. [https://doi.org/10.1016/S0955-2863\(99\)00083-2](https://doi.org/10.1016/S0955-2863(99)00083-2).
- Tagliacucchi, D., Martini, S., Bellesia, A., & Conte, A. (2015). Identification of ACE-inhibitory peptides from Phaseolus vulgaris after in vitro gastrointestinal digestion. *International Journal of Food Sciences and Nutrition*, 66(7), 774–782. <https://doi.org/10.3109/09637486.2015.1088940>.
- Tipton, K. D., Elliott, T. A., Cree, M. G., Wolf, S. E., Sanford, A. P., & Wolfe, R. R. (2004). Ingestion of casein and whey proteins result in muscle anabolism after resistance exercise. *Medicine & Science in Sports & Exercise*, 36(12), 2073–2081.
- Théolier, J., Hammami, R., Labelle, P., Fliss, I., & Jean, J. (2013). Isolation and identification of antimicrobial peptides derived by peptic cleavage of whey protein isolate. *Journal of Functional Foods*, 5(2), 706–714. <https://doi.org/10.1016/j.jff.2013.01.014>.
- Vermeirssen, V., Deplancke, B., Tappenden, K. A., Camp, J. V., Gaskins, H. R., & Verstraete, W. (2002). Intestinal transport of the lactokinins Ala-Leu-Pro-Met-His-Ile-Arg through a Caco-2 Bbe monolayer. *Journal of Peptide Science: An Official Publication of the European Peptide Society*, 8(3), 95–100. <https://doi.org/10.1002/psc.371>.
- Walrand, S., Gryson, C., Salles, J., Giraudet, C., Migné, C., Bonhomme, C., ... Boirie, Y. (2016). Fast-digestive protein supplement for ten days overcomes muscle anabolic resistance in healthy elderly men. *Clinical Nutrition*, 35(3), 660–668. <https://doi.org/10.1016/j.clnu.2015.04.020>.
- Wang, C., Wang, B., & Li, B. (2016). Bioavailability of peptides from casein hydrolysate in vitro: Amino acid compositions of peptides affect the antioxidant efficacy and resistance to intestinal peptidases. *Food Research International*, 81, 188–196. <https://doi.org/10.1016/j.foodres.2015.12.013>.