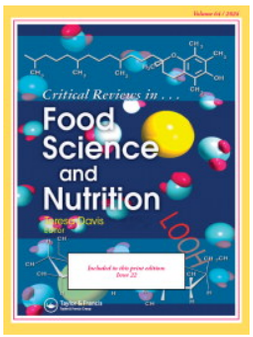

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REVIEW ARTICLE

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Coupling *in vitro* food digestion with *in vitro* epithelial absorption; recommendations for biocompatibility

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ABSTRACT

As food transits the gastrointestinal tract, food structures are disrupted and nutrients are absorbed across the gut barrier. In the past decade, great efforts have focused on the creation of a consensus gastrointestinal digestion protocol (i.e., INFOGEST method) to mimic digestion in the upper gut. However, to better determine the fate of food components, it is also critical to mimic food absorption *in vitro*. This is usually performed by treating polarized epithelial cells (i.e., differentiated Caco-2 monolayers) with food digesta. This food digesta contains digestive enzymes and bile salts, and if following the INFOGEST protocol, at concentrations that although physiologically relevant are harmful to cells. The lack of a harmonized protocol on how to prepare the food digesta samples for downstream Caco-2 studies creates challenges in comparing inter laboratory results. This article aims to critically review the current detoxification practices, highlight potential routes and their limitations, and recommend common approaches to ensure food digesta is biocompatible with Caco-2 monolayers. Our ultimate aim is to agree a harmonized consensus protocol or framework for *in vitro* studies focused on the absorption of food components across the intestinal barrier.

KEYWORDS

Caco-2; digesta detoxification; food absorption; INFOGEST

Introduction

The complex interplay between food components, the food matrix and the gastrointestinal (GI) tract determines the fate of food components as they transit the gut, their digestion, their bioaccessibility, their absorption and their bioefficacy.

In the past decade, great efforts have focused on developing means to mimic, *in vitro*, the breakdown of food as it transits the GI tract, by employing static, semi-dynamic and dynamic methods (Mackie, Mulet-Cabero, and Torcello-Gómez 2020). The INFOGEST method is an *in vitro* static method (Brodkorb et al. 2019; Minekus et al. 2014), with clearly defined salt concentrations, pH, enzymatic activities and dilutions for oral, gastric and upper intestinal phases. This consensus method was carefully designed to mimic as close as possible the physiological state of the GI tract of the healthy adult under fed conditions and, at the same time, be relatively inexpensive and employ standard laboratory equipment. The INFOGEST protocol has been validated with pig and human gut lumen data, particularly for proteins (Miralles et al. 2021; Sousa et al. 2023). As such, this protocol is regarded as the "best in its class" and is widely accepted internationally with more than 400 citations per year. Currently, adaptions to this standard method are being considered to more closely represent other life stages (Menard et al. 2018; Menard et al. Forthcoming) or to improve its relevance when following the digestion of minor food components, such as minerals (Muleya, Young, and Bailey 2021) or lipophilic bioactive compounds (i.e., sterols and carotenoids) (Makran et al. 2022; Petry and Mercadante 2020).

Although the INFOGEST protocol is well suited to determine the breakdown of food components in the upper GI tract, there is no consensus protocol to study the absorption of food components across the intestinal barrier. Studying

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the interplay between food digesta and the gut barrier *in vitro* is critically important primarily (a) to track the absorption of food components particularly for nutrition, safety and allergy assessments, (b) to investigate nutrient sensing, (c) to mechanistically evaluate the fate of bioactive components and their bioefficacy, (d) to evaluate potential interactions between food and pharmaceutical components during absorption, (e) to determine concentration dependencies, and provide recommendations on optimization of delivery systems, through food formulations and processes, (f) to evaluate the effect of digesta on intestinal barrier health parameters and (g) to collect the bioavailable fraction for assays on other cell models outside of the GI tract.

A well suited, physiologically relevant cell absorption model following the INFOGEST digestion protocol would help to avoid, or, at the very least, limit the requirement for animal studies.

In vitro absorption is routinely studied using differentiated monolayers of the human colonic epithelial cell line, Caco-2. Cultured on membrane inserts plates and following a well defined protocol (Hubatsch, Ragnarsson, and P. Artursson 2007), Caco-2 cells will differentiate over 21 days into polarized monolayers of absorptive enterocytes with tight junctions and limited paracellular diffusion (Fedi et al. 2021; Panse and Gerk 2022). This model is well-established and commonly employed as an initial screening tool for the evaluation of drug and food absorption, and to study components in isolation, to derive a mechanistic understanding of their bioefficacy (Fedi et al. 2021). There is a strong correlation (R^2 = 0.84) between permeability data generated from Caco-2 monolayers and from human trials for drug compounds utilizing transcellular passive transport (Jarc et al. 2019). However it is unrealistic to expect any single cell line to represent the morphology and absorption differences along the entire length of the human small intestine. There are notable differences between Caco-2 monolayers and the small intestine, not least of which includes tight junction diameter differences, the absence of a mucus layer, fluid flow and differences in transporter expression (Fedi et al. 2021; Franco, Da Silva, and R. Cristofoletti 2021). Moreover, the static Caco-2 model lacks the complex architecture and dynamic microenvironment present *in vivo* and in 3D models (Franco, Da Silva, and R. Cristofoletti 2021), making it more susceptible to damage. Furthermore comparative research is needed to investigate the correlation of data from Caco-2 monolayers to human trials, for food components (including lipophilic components) absorbed *via* paracellular diffusion or carrier mediated processes. However, unlike more complex models, this model allows for a harmonized approach to studying absorption for interlaboratory comparisons.

Caco-2 cells are often co-cultured with the human colon adenocarcinoma cell line HT-29 treated with methotrexate (HT-29MTX). This adds a mucus layer to the polarized monolayer which better mimics "real life" conditions (Hilgendorf et al. 2000), adding a higher level of complexity to the absorption, whilst still maintaining the ease of comparison between studies (Arranz et al. 2017; Hilgendorf et al. 2000). Alternatively, *ex vivo* mucus can be added to Caco-2 monolayers in the absence of HT-29MTX (Birch et al. 2018; Boegh et al. 2014). In addition, it has recently been demonstrated that Caco-2 monolayers can be modified with sodium butyrate to mimic the higher permeability of infant gut barriers (Kondrashina, Brodkorb, and Giblin 2021). Cell lines of human origin T84 (Devriese et al. 2017), SK-CO15 (Yoo et al. 2012) and HCT-8 (Hurley et al. 2016) can also be cultured as monolayers with tight junctions and are often considered when colonic barrier function is the subject of the study. Other cell lines are available which can be cultured as monolayers including porcine IPEC-J2 (Zakrzewski et al. 2013), PoCo83-3, ZYM-SIEC02 and bovine BIEC and FBCEC (Ghiselli et al. 2021). Primary cell cultures, more complex 3D and gut-on-a-chip models do exist. However to date there are very few studies where these models have been treated with digested foods.

Caco-2 monolayers remain a valuable tool to assess the interaction between digested food and the gut barrier. Culturing monolayers is cheap and relatively easy to do in a standard cell culture lab and the data generated is reproducible. Therefore this review focuses on studies coupling *in vitro* food digestion with Caco-2 or Caco-2/HT-29 co-cultures.

The challenge

Treating Caco-2 models *in vitro* with soluble food digesta stemming from the INFOGEST protocol requires some preparatory steps to ensure biocompatibility. To successfully grow and maintain Caco-2 monolayers requires adherence to a standardized protocol that defines passage number range, seeding densities, pH range, media composition and osmolality (Hubatsch, Ragnarsson, and P. Artursson 2007). The INFOGEST intestinal end point contains (1) active digestive enzymes which detach these monolayers, (2) bile salts which are toxic to cells and can shift osmolality and (3) food derived compounds generated during digestion which may also be harmful to these 2D monolayers.

Digestive enzymes

Trypsin is the most active protease in the intestinal phase and the most abundant enzyme of pancreatin. The INFOGEST protocol recommends that the concentration of pancreatin added should ensure 100 U/mL of trypsin activity (Brodkorb et al. 2019). However, a Trypsin/EDTA solution (approx. 0.1 mM trypsin) is routinely used in cell culture to detach adherent cells including Caco-2 (Lea 2015). It is widely accepted that direct treatment of Caco-2 polarized monolayers with INFOGEST-derived food digesta will destroy barrier integrity and detach monolayers. In addition, Darmoul et al. (2001) reported that 10 nM trypsin will detach HT-29 cells from their plastic supports.

Pepsin is the most active enzyme in the gastric phase with both protease and peptidase activities. The INFOGEST protocol recommends the use of 2000 U/mL gastric pepsin (Brodkorb et al. 2019). Reports on toxic effects of pepsin on Caco-2 monolayers are limited, however there are reports

in other cell lines. The human hypopharyngeal primary cells treated with pepsin (1 mg/mL (approximately 4000 U/mg) at pH 5.0) for 15 min daily for 5 days resulted in 48% decrease in cell viability compared to non-treated control (Doukas et al. 2021). This is in agreement with Hurley et al. (2019) who observed that pepsin (pH 3.0) at concentrations 3-100 µg/mL significantly reduced the viability of human lung epithelial cell line H292 over 1 h compared to cells in buffer.

The INFOGEST protocol recommends 60 U/mL gastric lipase in the gastric phase and 2000 U/mL pancreatic lipase in the intestinal phase (Brodkorb et al. 2019; Minekus et al. 2014). In the human, lipase activity ranges from 10-120 U/mL in the stomach and 80-7000 U/mL in the duodenal tract. Qiu et al. (2021) investigated the effect of pancreatic lipase on non-differentiated and differentiated Caco-2 monolayers. Viability of cells was reduced more than 2-fold over a 4 h incubation with 0.5 U/mL of this lipase compared to media without lipase (Qiu et al. 2021). Interestingly, lowering the lipase concentrations to 0.1 U/mL had a positive impact on confluent Caco-2 cells over a 12 h period by significantly increasing synthesis of tight junction proteins (Qiu et al. 2021).

Bile

In the lumen of the small intestine, concentration of bile salts increases from 3 mM to 20 mM from the fasted to the fed state (Brayden and Stuettgen 2021). The INFOGEST protocol recommends the addition of fresh/frozen bile or bile extract (preferably bovine) for the intestinal phase of digestion, to ensure 10 mM bile salt concentration. Typically, commercial bovine bile also contains phospholipids (e.g., lecithin), cholesterol, proteins and bilirubin.

Bile salts are not only required for GI digestion but also for the absorption of lipids and lipophilic components so they should be included in food absorption experiments. However, bile salt toxicity to Caco-2 cells is well known. Brayden and Stuettgen (2021) monitored viability of non-differentiated Caco-2 cells with sodium glycodeoxycholate and sodium deoxycholate over a 2 h period. An IC_{50} value of 2 mM for sodium glycodeoxycholate and 1.5 mM for sodium deoxycholate was reported (Brayden and Stuettgen 2021). A significant decrease in mitochondrial membrane potential was observed for glycodeoxycholate at concentrations >1.5 mM and for sodium deoxycholate at all tested concentrations (0.5-10 mM), compared to Caco-2 cells in Hanks Balanced Salt Solution (HBSS) buffer control (Brayden and Stuettgen 2021). Neither of these bile salts caused oxidative stress, but 1-1.5 mM sodium deoxycholate did increase activities of apoptotic biomarkers (Brayden and Stuettgen 2021). An earlier study by Patel et al. (2006) reported an IC_{50} value of 0.4 mM for sodium deoxycholate in non-differentiated Caco-2 for the same time period. Taurocholate was less toxic with an IC_{50} of 15 mM, while taurodeoxycholate, cholate and glycocholate had intermediate cytotoxicity (Patel et al. 2006). When tested on differentiated Caco-2 monolayers, 0.5 mM glycodeoxycholate in modified HBSS buffer decreased monolayer integrity measured as transepithelial electrical resistance (TEER) by 70%, from baseline ($2097 \pm 128\ \Omega \times cm^2$) within 20 min of treatment and compromised permeability as determined by ^{14}C-mannitol (Brayden and Stuettgen 2021). These treated monolayers in fresh media were unable to recover (Brayden and Stuettgen 2021). However, recovery of monolayers was observed if a lower concentration of glycodeoxycholate (0.1 mM) was used (Brayden and Stuettgen 2021).

Surprisingly the presence of phospholipids in bile can provide a protective effect to Caco-2 monolayers. Tan et al. (2013) observed that Caco-2 cells could tolerate mixed micelles containing 0.6 mM sodium deoxycholate in the presence of lecithin (1:2 ratio). However in the absence of lecithin, 0.2 mM sodium deoxycholate micelles significantly reduced cell viability ($p < 0.01$) (Tan et al. 2013). Ingels et al. (2002) treated Caco-2 monolayers with simulated intestinal solutions representing either a fasted or fed state. These intestinal solutions did not include digestive enzymes but did contain lecithin, sodium taurocholate, KH_2PO_4 and KCl at different concentrations and different pHs. For the simulated fasted solution, the addition of 1.5 mM lecithin, protected Caco-2 monolayers from permeability damage caused by 5 mM sodium taurocholate at pH 6.8 (Ingels et al. 2002). However, the fed simulated solution of 3.75 mM lecithin, 15 mM sodium taurocholate, 8.65 g/L KH_2PO_4 and 15.2 g/L KCl at pH 5.0 triggered a rapid decrease in TEER values in Caco-2 monolayers, falling below 10% within 15 min of control monolayers. Moreover, viability of Caco-2 monolayers was only 5.4% after 2 h treatment with fed simulated solution compared to the HBSS control (Ingels et al. 2002). The low pH (5.0), high concentrations of sodium taurocholate (15 mM) and high osmolarity (600 mOsm/L) in the fed simulated solution explains this toxicity even in the absence of digestive enzymes (Ingels et al. 2002).

Osmolarity and pH

Osmolarity and pH are not considered major concerns for the biocompatibility of INFOGEST fluids at the end of the intestinal phase. In the absence of food, the INFOGEST intestinal end point has an osmolarity of approx. 164 mOsm/L (De la Fuente et al. 2020). *In vivo* the osmolarity of the intraluminal fluid of the small intestine is approx. 285-300 mOsm/L. Caco-2 cells are isotonic at 336 mOsm/L (Grauso et al. 2019). However, the presence of food may alter the osmolarity of the INFOGEST intestinal end point. It is important to keep in mind that increasing osmolality up to 700 mOsm/kg leads to a dose-dependent decrease in TEER and an increase in Caco-2 monolayer permeability (Inokuchi et al. 2009).

The intraluminal pH in the small intestine varies according to the location with a gradual increase from 5.7-6.4 in the duodenum, to 7.4 in the jejunum and reaching 7.7 in the ileum. The INFOGEST protocol recommends pH 7.0 for optimal intestinal digestion (Brodkorb et al. 2019) which also lends the digesta suitable for subsequent Caco-2 assays. Sambuy et al. (2005) reviewed culturing conditions for

Caco-2 monolayers, stating pH had a significant influence on proliferation and differentiation, with optimum Caco-2 proliferation at pH 7.2 and significant differences in viability from pH 7.0 to 8.5.

Addressing the challenge

Despite high concentrations of digestive enzymes and bile salts, many laboratories have devised protocols to detoxify food digesta for addition to Caco-2 or other cell lines to study food absorption and/or bioactivity. It is important to review and summarize this literature, and evaluate best practices. We have limited our review, as far as possible, to methods used to detoxify digesta collected from INFOGEST static GI protocols. Reference to GI digestion methods that do not use the INFOGEST protocol are labeled as "Not-INFOGEST" (NI). Indeed, many studies simply elected to perform static GI protocols with lower concentrations of digestive enzymes (Muleya, Young, and Bailey 2021) and bile salts (Santos-Hernández, Amigo, and Recio 2020) than those recommended in the INFOGEST protocol. The substantial evidence in recent years confirms that the INFOGEST protocol correlates well with GI digestion *in vivo*, and therefore reducing concentrations of GI enzymes and bile salts for digestion limits the relevance of the experimental data, and should be avoided.

For this review, studies which included a "digesta control" (digestive solutions and enzymes without food) on Caco-2 cells were of particular interest as they would indicate the toxicity challenges brought by the digestive simulated juices. It is important to note that detoxification protocols are primarily selected by whether the study is tracking an individual food component or focused on all food components in the digesta. The main aim of this review is to create a series of recommendations to assist in detoxifying the soluble digesta derived from the static INFOGEST protocol for biocompatibility with Caco-2 monolayers.

Enzyme inhibitors

After the end of the intestinal phase, INFOGEST recommends the use of enzyme inhibitors followed by snap freezing to terminate GI digestion (Brodkorb et al. 2019). However, many studies simply state the INFOGEST protocol was performed and do not detail the type or concentration of enzyme inhibitor(s) used, if any. Our first recommendation is to state if an enzyme inhibitor was used and to include concentration. Table 1 details the commercial inhibitors available, their substrates, their optimum concentration for inhibition and their effects on cell lines. The choice of an inhibitor often depends on the macronutrient of interest in the food matrix and the time point of digesta sampling.

Protease inhibitors

When gastric samples are of interest and a pH increase is not an option, the aspartyl protease inhibitor, Pepstatin A, can be used (Rich et al. 1985). At 0.5-1 µM, Pepstatin A quickly and selectively inhibits pepsin in the digesta, with 0.72 µM reported as the optimum concentration (Egger et al. 2021). Moreover, Pepstatin A has limited side activities due to its low solubility in aqueous media (Van Kasteren et al. 2011). In addition, inclusion of a mucus layer artificially or *via* HT-29MTX co-culturing will no doubt give additional protection to differentiated Caco-2 monolayers (Antoine et al. 2015) against Pepstatin A.

Pefabloc inhibits serine proteases (Table 1) and the current recommendation by INFOGEST is to use a concentration of 5 mM at the end of the intestinal phase. Pefabloc at 0.17 mM was well tolerated by the human neuroblastoma SH-SY5Y cells (Klegeris and McGeer 2005). However, 0.6 mM Pefabloc resulted in a 60% reduction in metabolic activity in kidney fibroblast-like Cos7 cells (Mao et al. 2003). With immature 4 day old Caco-2 monolayers, Buzza et al. (2010) reported that 0.025 mM Pefabloc added to cultured media can significantly curtail barrier development. Abdel-Aal et al. (2023) added 1 mM Pefabloc post NI-GI digestion of bread and muffins. The food digesta was then diluted 1 in 4 using DMEM media, equivalent to 0.25 mM Pefabloc. Caco-2 monolayer detachment was observed after 4 h treatment with digesta, with or without Pefabloc (Abdel-Aal et al. 2023). Whether Pefabloc contributed to this detachment was not investigated. Regardless, the evidence with Caco-2 cells suggests care must be taken with the final Pefabloc concentration present in monolayer assays. Recently several studies (Arranz et al. 2023, Egger et al. 2021, Zenker et al. 2020) have used lower concentrations of Pefabloc (1 mM or 0.5 mM) to terminate digestion at the end of the INFOGEST intestinal phase. Indeed supplier information sheets indicate that Pefabloc at a concentration of 0.5 mM is sufficient to inhibit chymotrypsin (Roche Diagnostics GmbH 2020). Therefore there is scope to reduce the concentration of Pefabloc used at the end of the INFOGEST protocol, with a further dilution probably required prior to Caco-2 assays.

Legumes-derived Bowman–Birk Inhibitors are plant-based extracts composed of serine protease inhibitors that effectively inhibit trypsin and chymotrypsin (Table 1), contributing to the anti-nutritional characteristics of plants (Gitlin-Domagalska, Maciejewska, and Dębowski 2020). Bowman–Birk Inhibitors are rich in disulfide bonds and form stable complexes with proteases to inhibit their activity. To date there is limited use of Bowman–Birk Inhibitors in static GI digestion protocols. Cruz-Huerta et al. (2015) observed that even when GI digested and heat inactivated, Bowman–Birk Inhibitors can still decrease cell viability in both HT-29 and Caco-2 cells over a 24 h period at concentrations > 0.05 mg/mL, albeit the assessment was performed in the presence of the soy peptide, lunasin.

Lipase inhibitors

Tetrahydrolipstatin (Orlistat) is a specific inhibitor of gastric and pancreatic lipases (Table 1). It forms a covalent bond with the active serine site of lipases, which prevents hydrolysis of triglycerides (Heck, Yanovski, and Calis 2000). The INFOGEST protocol recommends a final concentration of

Table 1. Inhibitors of digestive enzymes.

Type	Name & activity	M_W & Source	Final recommended concentration	Targeted enzymes	Expected effect on follow up cells-based assays	Expected effect on cell monolayers and endogenous enzymes	Reference
Protease/ Peptidase inhibitor	Pepstatin A - inhibitor of asparatyl peptidases	685.89 g/mol. Sequence: *Iva-Val-Val-Sta-Ala-Sta* Actinomycetes (first isolated), synthetic.	0.5-1 µM 0.72 µM (optimum)	Pepsin M_W = 34.55 kDa (porcine)	Low solubility (in water) and cell penetration → low interference. 0.5-1 µg/ml: ↓ CD4 cell proliferation (day 7) and inflammation (IL-2 and IFN-γ) in tonsil tissue stimulated with 1 µg/ml acid pepsin	Not known	Kim et al. 2018
	Pefabloc SC -inhibitor of serine proteases	239.5 g/mol 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), synthetic.	0.5-5 mM	Trypsin, chymotrypsin M_W = 23.44; 25.6 kDa (porcine)	0.17 mM: no cytotoxicity on SH-SY5Y cells (2×10^5 cells/mL), 72 h, MTT assay 0.31 mM: no cytotoxicity on undifferentiated Caco-2 and HT29-MTX cells (1×10^4 cells/well – 96-well plates), 24 h, MTT assay. Still toxic at 0.62 mM final concentration. 0.6 mM: 60% ↓ metabolic activity in Cos7 cells, 2 h, alamarBlue™	25 µM significantly ↓ TEER values vs. untreated (500 vs. 1750 Ω ×cm²) differentiated Caco-2, 8 days treatment Expected ↓ activity of intestinal brush border peptidases.	Buzza et al. 2010; Klegeris and McGeer 2005; Mao et al. 2003
	Bowman–Birk inhibitor (BBI) - inhibitor of serine proteases	8-16 kDa from monocotyledonous plants, 6-9 kDa from dicotyledonous plants. Plant origin	5 mg/L	Trypsin, chymotrypsin M_W = 23.44; 25.6 kDa (porcine)	1:1, 1:2 food peptide to BBI ratio: No cytotoxicity of co-digested BBI to 7×10^4 cells/cm² Caco-2 and HT29 cells at 0.1 mg/mL protein by MTT, 24 h. Cytotoxic at 0.2 and 0.3 mg/mL protein. 100 µg/mL: ↓ IL-1β, TNF-α and IL-6 secretion from 24 h pretreated human macrophages activated with LPS (100 ng/mL)	Expected ↓ activity of intestinal brush border peptidases	Basson et al. 2021; Cruz-Huerta et al. 2015
Lipase inhibitor	Orlistat - inhibitor of lipases	495.73 g/L Microbial origin	0.6 mg/mL (1 mM) 3 mg/mL (5 mM)	Gastric lipase M_W = 50 kDa (rabbit) Pancreatic lipase M_W = 48 kDa (porcine)	100 µM: significantly ↓ GAPDH mRNA transcripts in HT29 cells over 48 h. Not toxic to Caco-2 in the same conditions.	1µM: no cytotoxicity in differentiated (14-21 days) Caco-2 monolayers (stable TEER > 1000 Ω×cm²) Expected ↓ activity of intestinal brush border lipase	Alhamoruni et al. 2012; Martin et al. 2013; Spalinger et al. 1998
	4-bromo-phenylboronic acid - inhibitor of lipases	200.83 g/L Synthetic	5 mM	Pancreatic lipase M_W = 48 kDa (porcine)	Not known	2.5 mM: no ↓ P_{app} of mannitol in differentiated (21-26 days) Caco-2 monolayers. Expected ↓ activity of intestinal brush border lipase	Keemink and Bergström 2018; Spalinger et al. 1998

Abbreviations: AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; BBI, Bowman–Birk inhibitor; GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase; IL-2, Interleukin-2; IFN, Interferon; TNF, Tumour Necrosis Factor; IL-6, Interleukin-6; IL-1, Interleukin-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; TEER, Transepithelial Electrical Resistance.

1 mM Orlistat to inhibit gastric lipase and of 5 mM to inhibit intestinal lipase. Orlistat effectively inhibits gastric lipase at an inhibitor to enzyme molar ratio of 1 in 2,000 (Grundy et al. 2021). For pancreatic lipase, inhibition has been described at an Orlistat to enzyme molar ratio of 1 in 10,000, although this inhibitory effect is reversible (Gargouri et al. 1991; Grundy et al. 2021). The toxicity of Orlistat has been assessed *in vitro* using cell lines. Browne, Hindmarsh, and Smith (2006) reported an Orlistat IC_{50} of 1-3 µM in HUVECs endothelial cells. Orlistat may be more damaging to HT-29 cells than Caco-2 cells with Martin et al. (2013) reporting significantly lower GAPDH mRNA transcripts in HT-29 cells treated with 100 µM Orlistat for 48 h in media. This decrease was not observed in Caco-2 cells (Martin et al. 2013). At lower concentrations, Orlistat may even act to protect Caco-2 monolayer integrity, as Alhamoruni et al. (2012) observed that the addition of Orlistat (1 µM) together with cytokines to the apical chamber of Caco-2 monolayers, prevented the TEER reduction normally associated with cytokine application alone.

An alternative inhibitor of pancreatic lipase is 4-bromophenylboronic acid (Table 1). A 4-bromophenylboronic acid to pancreatic lipase molar ratio of 1 in 14,300, significantly inhibits enzyme activity. Grundy et al. (2021) recommended the addition of 4-bromophenylboronic acid at a concentration of 5 mM to intestinal digests for optimal irreversible inhibition of pancreatic lipase. However, 4-bromophenylboronic acid is not an effective inhibitor of gastric lipase, as a high remaining activity of gastric lipase was found even at an inhibitor to lipase molar ratio of 1 in 13,500 (Grundy et al. 2021). In addition, it's cytotoxicity on cell lines is not well established (Table 1).

Amylase inhibitors

Amylase activity is effectively inhibited by 12% (wt/vol) trichloroacetic acid precipitation (Brodkorb et al. 2019; Villemejane et al. 2016). However, such treatment precipitates protein, which may not be desirable for downstream cell absorptions assays. If trichloroacetic acid is used, a concentration of 1.2 mg/L trichloroacetic acid will not cross 21 day old Caco-2 monolayers after a 2 h treatment in HBSS (Melo et al. 2016). In addition, a combination of 5 haloacetic acids (including trichloroacetic acid at 1.5 mg/L final concentration) in DMEM is not cytotoxic to Caco-2 cells after 2 h (Melo et al. 2016).

Temperature inactivation

A popular and simple alternative to enzyme inhibition is heat inactivation of digestive enzymes. Jovani et al. (2001) recommended heating for at least 4 min at 100 °C. This is suitable where the bioavailability of minerals (Jovani et al. 2001), polyphenols (Ben Hlel et al. 2019) and oils (Seiquer et al. 2015) are under investigation post digestion. However, the major drawbacks of heat inactivation is that the temperature employed can cause (1) an irreversible denaturation and aggregation of residual proteins (Joyce, Kelly, and O'Mahony 2018), (2) formation of advanced glycation end products where sugars and peptides are present (Sun et al. 2022), (3) destruction of heat labile compounds such as vitamins (Lalwani et al. 2021) and (4) oxidation of lipidic compounds such as carotenoids or sterols (Garcia-Llatas and Rodriguez-Estrada 2011). In addition, heat treatment of the digesta is not suitable where the effect of thermal processing on food is under investigation (Garcia-Llatas and Rodriguez-Estrada 2011).

pH

Where gastric samples are of interest for cell studies, increasing pH from 3.0 (INFOGEST method) to pH 7.5 with sodium hydroxide or sodium bicarbonate will halt pepsin activity. However, trypsin exhibits activity at pH 6 to 9 and stability from pH 4 to 11 (Klomklao et al. 2009) so pH adjustment alone is unlikely to inhibit trypsin in intestinal samples. Where polyphenols were tracked, Cilla et al. (2009) added 1.5% formic acid to decrease the pH of samples post intestinal digestion to pH 2. The samples were then diluted in media not only to reach a pH of 7.0-7.5 but also the addition of media would inhibit any reversible trypsin activity (Cilla et al. 2009).

Dilution with cell culture media and buffer

Dilution of food digesta in cell culture media containing fetal bovine serum (FBS) is a popular choice to dilute bile salts and inactivate digestive enzymes. For bile salts, Brayden and Stuettgen (2021) provided evidence that barrier integrity of Caco-2 monolayers is not damaged with a glycodeoxycholate concentration below 0.1 mM. Therefore based on the concentration of glycodeoxycholate in bovine bile (Hu et al. 2018) and the protective effect of phospholipids, a 1 in 10 dilution of digesta is a recommended starting point.

It is difficult to recommend a dilution factor and diluent that would ensure digestive enzymes did not damage monolayers, as results from different studies are equivocal. The inclusion of a digesta control in all future studies would add clarity. FBS contains alpha-1 antitrypsin which inactivates trypsin (Tang, Wang, and Liao 2021; Stockley 2015). Post NI-GI digestion and to evaluate calcium transport in Caco-2 cells, Ekmekcioglu et al. (1999) simply added 1% v/v of FBS serum to mineral water digesta. This addition allowed 15 day old Caco-2 monolayers to tolerate the digesta for 90 min without showing signs of monolayer impairment or brush border enzyme toxicity (Ekmekcioglu et al. 1999). In another study, β-carotene rich micelles post NI-GI digestion were collected by ultracentrifugation (Corte-Real et al. 2014). Caco-2 monolayers (14 day old) were treated for 4 h to this aqueous micellar phase diluted in DMEM with 20% FBS. Cellular viability was similar to media control at a dilution factor of 1 in 4 (Corte-Real et al. 2014). Guri, Haratifar, and Corredig (2014) incubated undifferentiated HT-29 cells for 2 h with digestive fluids diluted in DMEM plus 10% FBS from 1 in 3 to 1 in 33 v/v. Dilutions lower than 1 in 9 caused a significant reduction (30-80%) in cell viability compared to cells in media which guided the researchers to proceed with 1 in 17 dilution to ensure viability of >80% (Guri, Haratifar, and Corredig 2014). Jilani et al. (2020) reported a minor decrease in undifferentiated Caco-2 cell proliferation

(3-15%) when treated with control digesta (NI-GI digestion fluids plus enzymes) diluted 1 in 3, 1 in 4 and 1 in 9 v/v with DMEM plus 10% FBS for 2-24 h. This was in agreement with Cilla et al. (2009) who observed 12% decrease in undifferentiated Caco-2 cell viability with control digesta diluted as 2, 5 and 7.5% v/v in Eagles Minimal Essential Media (EMEM) plus 10% FBS culture media compared to untreated cells in media alone. Likewise, treatment of the human colonic HCT-116 or Caco-2 undifferentiated cells to control GI digesta diluted in DMEM were not cytotoxic at 24 and 48 h time points (Cilla et al. 2022). The dilutions used were 1 in 11 and 1 in 21 v/v with DMEM containing 10% FBS (Cilla et al. 2022). Sabouri et al. (2018) cooled epigallocatechin-gallate emulsion (7% soybean oil) samples immediately after GI digestion and then diluted the samples (1 in 27) with DMEM plus 10% FBS. There was no significant decrease in undifferentiated Caco-2 viability with digesta control compared to cells cultured in media alone (Sabouri et al. 2018). Food can make a significant contribution to toxicity. GI digested emulsions containing 7% canola oil and 5 mg/mL of rosemary supercritical extract were toxic to undifferentiated Caco-2 cells necessitating a substantial dilution of 1 in 61 with DMEM containing 10% FBS before proceeding to Caco-2 monolayer experiments (Arranz et al. 2017).

Other studies have reported that dilution in media without FBS can be used for Caco-2 studies (da Paixão Teixeira et al. 2022; Markell et al. 2017). Markell et al. (2017) combined heat inactivation with dilution in FBS free media to detoxify food digesta. Heat inactivated digesta was lyophilized and resuspended in FBS free media. There was no reduction in Caco-2 monolayer TEER values, viability or permeability with NI-GI digesta for 48 h compared to control (Markell et al. 2017).

The criteria to select a media dilution is balanced between cell viability of >/= 85-90% whilst simultaneously allowing the detection and ideally the quantification of the food component/derivative of interest. The main disadvantages of dilution with media are that it not only dilutes the food component of interest making it difficult to track but it also adds a layer of complexity to the digesta simply by its inherent nutritional composition. Potential interference by media has led many researchers to dilute digesta with buffers for Caco-2 monolayer experiments run over a short period of time, typically 2-4 h (Arranz et al. 2023, Bavaro et al. 2021; Corrochano et al. 2018). Dilution in buffer will dilute bile salts and adjust osmolarity but will not inactivate digestive enzymes. Therefore, studies routinely include an enzyme inactivation process before a buffer dilution step (Arranz et al. 2023; Bavaro et al. 2021; Corrochano et al. 2018; Faria, Melo, and Ferreira 2020; Sangsawad et al. 2018).

Physical separation

Many studies include physically removing the insoluble digesta, digestive enzymes and/or bile salts by centrifugation, filtration or dialysis.

Centrifugation

Faria, Melo, and Ferreira (2020) included a blank digesta control in their study to track food contaminants. After *in vitro* GI digestion, the blank digesta was spiked with contaminants, vortexed for 5 min, centrifuged at 12,000 g at 4 °C for 45 min to collect the supernatant. Successful transport assays across Caco-2 monolayers were performed for 2 h using a 1 in 4 HBSS buffer dilution (Faria, Melo, and Ferreira 2020). Santos-Hernández et al. (2021) employed a modified INFOGEST protocol (2.5 mM bile) on whey and egg protein. Post GI digestion, the test samples were heated to 85 °C for 15 min and then centrifuged at 5000 g for 15 min to pellet the insoluble fraction. No adverse effects on TEER values were reported over a 2 h period with the soluble digesta (2 mg/mL protein) diluted in buffer (Santos-Hernández et al. 2021). Black carrot and its by-products were NI-GI digested, centrifuged at 3000 g at 4 °C for 10 min and the supernatants collected and dried (Kamiloglu et al. 2017). In this case, monolayer TEER values fell over a 4 h period by 30% and 18% when incubated with digesta control resuspended in buffer or media respectively (Kamiloglu et al. 2017). Yao et al. (2020) tracked the bioavailability of phenolic compounds across Caco-2 monolayers post NI-GI digestion of buckwheat. Digesta was centrifuged at 16,770 g for 10 min, mixed 1:1 with 80% acetone and centrifuged again at 16,770 g for 10 min to collect the polyphenol fraction. Caco-2 monolayers could tolerate this fraction (2.5 mg/mL polyphenol) in HBSS for 120 mins (Yao et al. 2020). Do Nascimento et al. (2021) investigated the bioaccessibility of carotenoids extracted from *Scenedesmus obliquus*. Post GI digestion, the digesta was centrifuged at 8,000 g at 4 °C for 60 min. The supernatant containing the mixed micelles was then diluted 1 in 4 with DMEM and added to Caco-2 monolayers for 4 h (do Nascimento et al. 2021).

Several studies have employed ultracentrifugation, often because the nutrient of interest was fat soluble or encapsulated. Di Silvio et al. (2016) included a sucrose gradient ultracentrifugation step at 195,000 g, 20 °C for 1 h. O'Callaghan and O'Brien (2010) added an ultracentrifugation step of 200,000 g for 95 min and Corte-Real et al. (2014) included a 164,000 g for 35 min at 4 °C ultracentrifugation step. Neither O'Callaghan's nor Di Silvio's studies observed a reduction in Caco-2 monolayer TEER values after 4 h incubation with supernatants in FBS free media. Such prolonged high speed centrifugation will remove a large fraction of digesta and is not suitable where all food components are under investigation.

Dialysis

Dialysis membranes with 10-15 kDa molecular weight cut offs (MWCO) will retain digestive enzymes, such as porcine trypsin (23.3 kDa), chymotrypsin (25.6 kDa), α-amylase (55.4 kDa), pancreatic lipase (55 kDa) and pepsin (35 kDa) (EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP) et al. 2022). Dialysis can also serve to desalt the digesta. Dialysis has been employed to study dietary iron uptake by Caco-2 monolayers using a dual chamber system. Glahn et al. 1998 performed a NI-intestinal digestion in the upper chamber of a two-chambered system with a 15 kDa MWCO dialysis membrane acting as the divide (Glahn et al. 1998). After a 2 h digestion, the upper chamber and dialysis insert were removed. The Caco-2

monolayers in the bottom chamber were treated with the bioaccessible fraction for a further 22 h. Toxicity was not tested but as cell lysate was collected any loss of monolayers would have been noted (Glahn et al. 1998). Lingua et al. (2019) employed a 10 kDa MWCO dialysis bag to perform a 2 h intestinal digestion of fresh grapes and wine. Subsequently non-differentiated Caco-2 cells treated with the dialyzed fraction, at 20 ng/mL polyphenols in DMEM, had similar viability to cells in media alone (Lingua et al. 2019).

It is important to note that for mineral studies, dialysis may retain peptide carriers. Dialysis should therefore be avoided where peptide-mineral interactions are of interest. In general, dialysis is not recommended, as it can result in dilution of the compound of interest. For example, Jovaní et al. (2001) performed a GI digestion of infant formula within a dialysis bag (12 kDa MWCO). However, the sample was deemed too dilute to proceed to Caco-2 mineral uptake assays so the researchers favored a heat inactivated digesta sample as an alternative (Jovaní et al. 2001).

Ultrafiltration

Ultrafiltration with MWCO is often used to track a food bioactive of interest. Sangsawad et al. (2018) performed sequential ultrafiltration steps on NI-GI digested chicken protein using MWCO of 10 kDA, 3 kDA and 1 kDA. None of the peptide fractions, at 15 mg/mL in EMEM, altered Caco-2 viability over a 24 h period (Sangsawad et al. 2018). Another study describes the generation of peptide fractions from GI digestion of foxtail millet (*Setaria italica*) protein isolate using 3 kDa MWCO filter followed by Reversed-Phase High-Performance Liquid Chromatography (Hu et al. 2020). These peptide fractions (25-100 µg/mL in FBS free DMEM) did not alter viability of non-differentiated Caco-2 cells over a 24 h period compared to media alone (Hu et al. 2020). da Paixão Teixeira et al. (2022) collected fractions < 30 kDa from GI digested cheese. To ensure 85-90% Caco-2 viability, the filtrate was diluted 1 in 3 with DMEM without FBS (da Paixão Teixeira et al. 2022).

Other studies have used MWCO to separate nanoparticles from digestion enzymes. Aguilera-Garrido et al. (2022) utilized centrifugal filters of 100 kDa MWCO to collect solid lipid nanoparticles in the retentate and remove enzymes, bile salts and enzyme inhibitors after GI digestion. Collected nanoparticles in DMEM were not toxic to Caco-2 monolayers (Aguilera-Garrido et al. 2022). There are some reports of NI-GI digestions performed using immobilized digestive enzymes as an alternative to physical removal and although the digesta control is well tolerated by Caco-2 monolayers, the extent of digestion can be compromised (Keemink and Bergström 2018).

It should be noted that it is good practice to filter food digesta samples with 0.22 or 0.45 µm filters ensuring the removal of bacteria prior to cell culture work. This is not regarded as a detoxification step but it is an important step and should be stated. The main advantages and disadvantages of all detoxification strategies are summarized in Table 2.

Other adjustments

Post GI digestion, we recommend that test samples are checked for osmolality and adjusted, if necessary, prior to cell absorption experiments. Indeed, many laboratories report on the need to adjust osmolality prior to Caco-2 transport studies (Corrochano et al. 2019; Ekmekcioglu 2002; Jovaní et al. 2001). In addition, digesta pH may need to be adjusted depending on the cellular study. Where brush border enzyme activity is important, a pH closer to 8.0 is preferred as enzyme activity is lower at pH 7.0 (Sambuy et al. 2005). For iron bioavailability studies a pH of 6.2-6.7 may be physiologically relevant (Ekmekcioglu 2002), as iron absorption occurs mainly in the duodenum.

Detoxification of colonic samples for cellular assays

Absorption studies using Caco-2 monolayers have also been reported with samples generated from *in vitro* colonic fermentations. There are several *in vitro* colonic fermentation models available, from simple batch systems to more complex multistage continuous and controlled bioreactors, all with the common objective of cultivating a complex intestinal microbiota (Li and Zhang 2022). Samples from these bioreactors will contain microbiota, microbial metabolites and may contain toxins, undigestible and biotransformed food components. No consensus protocol exists to detoxify these samples for cellular assays. However, the majority of protocols will include centrifugation of the fermentation digesta to remove particulate matter (4500-5400 rpm, 10-30 min, 4 °C), filtration to remove microbiota (0.22-0.45 µm) and then dilution in culture media (from 1 in 2.5 to 1 in 200) (Agudelo et al. 2020; Caicedo-Lopez et al. 2021; Glei et al. 2021; Schlörmann et al. 2020).

Importance of digesta control

A chief recommendation is the inclusion of a digesta control in cellular assays. This allows the study to distinguish between potential toxic effects of the intrinsic digestive juices and the food component. We would recommend that absolute values for the digesta control and for the media be reported in cell experiments. Cilla et al. (2009) employed a digesta control defined as ultrapure H_2O subjected to NI-GI digestion with active enzymes. This control was centrifuged at 3890 g for 60 min at 4 °C to collect the soluble fraction. Digestive enzymes were inactivated by addition of formic acid and filter sterilized. This control was then diluted 7.5% in EMEM. An osmolarity range of 280-330 mOsm/L and a pH of 7.0-7.5 was recorded. Undifferentiated Caco-2 cells were treated with this sample for 4 h daily over 4 consecutive days (Cilla et al. 2009). A 12% reduction in Caco-2 proliferation was observed compared to cells with media alone. However, cell cycle distribution of Caco-2 cells in G0/G1 and G2/M phases were similar when treated with this control digesta or media over the 4 days (Cilla et al.

Table 2. Digesta detoxification options.

Procedure	Conditions	Pros	Cons
Enzyme inactivation			
Inhibitors	0.72 µM Pepstatin A / 0.5-5 mM Pefabloc SC / 5 mg/L Bowman–Birk inhibitor (BBI); 1-5 mM Orlistat / 5 mM 4-bromo-phenylboronic acid	Enzymes inactivated in cell culture conditions No change to concentration of compounds of interest No change to structure/composition of digesta Can be used for sensitive quantification (clean system)	Non physiological Interferes with Caco-2 brush border enzyme digestion
Temperature	4-10 min at 100 °C after gastric or intestinal phase	Irreversible enzyme inactivation No change to concentration of compounds of interest Can be used for sensitive quantification (clean system)	Non physiological Degradation of heat labile compounds Protein aggregation, glycation Oxidation of carotenoids and sterols
pH	Up to 6.5-7.5 with NaOH or $NaHCO_3$ to inactivate pepsin after gastric digestion	Physiological Inherent part of the digestion protocol Can be used for sensitive quantification (clean system) Popular for phenolic compounds	Not sufficient on its own
Dilution with media/buffer	Reconstitute freeze-dried digesta powders	Cheap, easy and popular Can be used for sensitive quantification (clean system) No change to structure of digesta	Often not sufficient on its own Sharply decreases the quantity of analytes Provides only dilution effect for enzymes
Dilution with FBS media	1:3 – 1:61 dilution with cell culture medium containing 10-20% FBS	Cheap, easy and popular Acts as substrate for proteases, effectively reducing their activity No change to structure of digesta	Sharply decreases the quantity of analytes Provides only dilution effect for lipases and amylases High concentration of FBS is non physiological and expensive Cannot be used for sensitive quantification (clean system)
Enzyme physical separation			
Centrifugation	Centrifugation: 3000-16770 *g*, 10-60 min, 4 °C	Cheap and easy Can be used for sensitive quantification (clean system) Standard laboratory centrifuges	Not sufficient on its own Removes larger compounds from digesta Needs further adjustments, as dilution, etc.
Dialysis	10-15 kDa cutoff membranes, pH = 7, 2-4 h, 37 °C	Reliably removes enzymes of larger than cutoff size No change to structure of digesta	Removes larger compounds from digesta Sharply decreases the quantity of analytes Cell culture insert format is not commercially available Time consuming
Cutoff membrane ultrafiltration	10-30 kDa centrifugation units	Reliably removes enzymes of larger than cutoff size Cheap, quick, easy and popular Standard laboratory centrifuges Can be used for sensitive quantification (clean system)	Limited volume can be processed Removes larger compounds from digesta Could require dilution prior to centrifugation

Abbreviations: FBS, Foetal Bovine Serum.

2009). In addition, critical regulators of mitosis, cyclin B1 and D, were similar in Caco-2 cells treated with either control digesta or media alone (Cilla et al. 2009). There was also no indication of Caco-2 apoptosis with control digesta (Cilla et al. 2009).

Jilani et al. (2020) also generated a similar digesta control i.e., ultrapure H_2O in NI-GI digestion procedure. The intestinal digestion was stopped by incubation on ice for 10 min, followed by centrifugation for 1 h at 3500 g at 4 °C. The supernatant was diluted 1 in 4 with DMEM to achieve pH 7.0-7.5 and osmolarity of 280-330 mOsm/L. Proliferation of undifferentiated Caco-2 was decreased with this digesta control by 8 +/-2% for 2 h or 15 +/- 8% for 24 h, compared to cells in media alone. In agreement with Cilla et al. (2009), the cell cycle was unaffected when the cells were exposed to control digesta (Jilani et al. 2020). De la Fuente et al. (2020) also reported a 10-12% reduction in Caco-2 cell viability with a GI digesta control compared to media alone. The digesta control was generated using H_2O as a substitute for food in the GI protocol. The digesta was centrifuged for 5 min at 4000 rpm, supernatants filter sterilized and then diluted 1 in 10 with DMEM (De la Fuente et al. 2020). Cruz-Huerta et al. (2015) reported viabilities of 70.5% +/- 8% for Caco-2 and 86.6 +/- 4% for HT-29 with digesta control over a 24 h period compared to media alone. For Caco-2 cells this could be improved to 100% by a 1.5 dilution in media supplemented with FBS (Cruz-Huerta et al. 2015).

Whether the digesta control should be generated with active or inactive digestive enzymes is controversial. In the absence of food, active enzymes will be subject to autolysis, resulting in the release of free amino acids and peptides which could potentially confound results. To avoid this, the digesta control could be generated by using inactive enzymes. Another possibility and potentially more relevant would be the inclusion of a customized neutral food sample for the study under investigation, akin to the non-protein cookie

recently used in INFOGEST protein digestion studies (Sousa et al. 2023).

Special circumstances resulting in INFOGEST protocol modification

Interference with food allergy assessments

When studying the absorption of food protein allergens, the relatively high buffer volumes used in the INFOGEST protocol is often problematic. Pure allergens can be extremely difficult and expensive to source, necessitating the use of smaller digestion volumes (Deng et al. 2020). Epithelial translocation of intact food allergens is likely to occur in the proximal small intestine (Heyman et al. 1988; Wheeler et al. 1993), so the time allocated by INFOGEST for intestinal digestion phase may not be appropriate. In fact *in vitro* GI digestion procedures are often excluded when testing allergens in cellular assays. However, we would recommend the use of at least a gastric digestion phase prior to exposing intestinal epithelial cell models to allergens. Where intestinal digestions are performed, it is important to note that pancreatin contains bacterial lipopolysaccharides which will confound studies investigating the response of epithelial and immune cells to the allergen. Many studies therefore replace pancreatin with individual digestion enzymes (Di Stasio et al. 2020). Caution should also be exercised when employing the detoxification steps of heat treatment, filtration or dialysis steps to digesta as proteolytic resistant allergens may be immunologically inactivated or removed with the GI enzymes. Considerable additional studies are required to combine GI digestion protocols with allergen research.

Protecting brush border enzymes

Some studies may need to preserve the enzyme activities of the brush border membrane during Caco-2 absorption experiments. Brush border membrane enzymes play an important role in food metabolism in the GI tract since they are responsible for the final stage of digestion (Holmes and Lobley 1989). Differentiated Caco-2 polarized monolayers express, on their apical side, brush border membrane enzymes for example peptidases, alkaline phosphatase and disaccharidases (Howell, Kenny, and Turner 1992). In general, the profile of intestinal hydrolases of differentiated Caco-2 is comparable to jejunal enterocytes (Ölander et al. 2016.), albeit the activities are lower (Chantret et al. 1994). Several studies have observed Caco-2 brush border enzyme digestion prior to peptide absorption. For instance, Quirós et al. (2008) reported that β-casein peptide LHLPLP is hydrolyzed to HLPLP *in situ* prior to transport across 21 day old Caco-2 monolayers. Miguel et al. (2008) revealed that the egg peptides FRADHPFL and YAEERYPIL are digested into shorter fragments immediately prior to their absorption across 21 day old Caco-2 monolayers. Similarly, Lacroix et al. (2017) found that bioactive whey peptides are highly degraded by peptidases present at the apical side of 21 day old Caco-2 monolayers. Picariello et al. (2013) observed that a β-lactoglobulin peptide harboring a potential IgE allergen epitope, was resistant to digestion but was partially degraded at the N-terminal end when transported across 18 day old Caco-2 cell monolayers. Therefore for peptide absorption studies, we would recommend that peptidase inhibitors are not used to detoxify digesta as these inhibitors are likely to inhibit endogenous Caco-2 brush border enzymes. Ding et al. (2015) treated 21 day old Caco-2 monolayers with the dipeptidyl peptidase 4 inhibitor, Diprotin A (1 mM), for 30 min prior to 2 h transport studies with egg white peptide RVPSL (5 mM). The study observed that brush border peptidases degraded 36% of the peptide in the apical chamber. However, pretreatment with Diprotin A reduced brush border peptide degradation to 23% (Ding et al. 2015).

Where brush border digestion is important and reliance on Caco-2 enzyme digestion is not sufficient, there is the option to add a brush border digestion step, using membrane extracts, at the end of the INFOGEST protocol (Di Stasio et al. 2020; Mamone and Picariello 2023; Vivanco-Maroto et al. 2022), prior to proceeding to absorption studies.

Interference with food quantification

Blanco-Morales et al. (2018) reported that the addition of porcine bile salts confounds cholesterol tracking during GI digestion. As such the study recommended reducing the bile concentration for intestinal digestion to 1.4 mM or replacing porcine bile with bovine bile. Muleya, Young, and Bailey (2021) reported intrinsic levels of iron and zinc in pancreatin (78 +/-0.1 mg/kg and 253 +/- 3.4 mg/kg respectively) and bile (111 +/- 6.7 mg/kg and 10.3 +/-1.6 mg/kg, respectively) which interfered with tracking the bioaccessibility of these minerals. As such the amount of pancreatin added at the intestinal phase was based on total proteolytic activity rather than trypsin activity alone, reducing the overall amount required. However pancreatin and bile salts still had considerable mineral binding capabilities, binding 35% and 53% spiked iron and zinc isotopes (Muleya, Young, and Bailey 2021).

Methods used to verify biocompatibility

Prior to absorption studies, researchers routinely test the success or failure of their "detoxification" protocols by employing cell viability assays which can assess cell wall integrity by enzyme leakage (e.g., lactate dehydrogenase), metabolic activity (e.g., 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) or MTT), membrane permeability (e.g., Trypan blue), cell proliferation (e.g., BrdU), apoptosis (e.g., annexin or catalase) or redox damage (e.g., reactive oxygen species or glutathione) (Table 3).

Although biocompatibility assessments should ideally be performed on polarized monolayers, many studies routinely test digesta on undifferentiated Caco-2 cells in 96 well plate formats (Table 3), with an assumption that viability data

Table 3. Effect of simulated gastric and intestinal fluids on cell viability.

SGID model	Detoxification method	Cell line	Culturing Conditions	Exposure time	Cytotoxicity assay	Cytotoxicity Result (cell viability %)	Reference
INFOGEST	Dilution in HBSS: 1 in 3 total of a 10 kDa permeate of digesta treated with AEBSF inhibitor and snap frozen	Caco-2/ HT-29	Caco-2: DMEM with 4.5 g/L glucose and 0.581 g/L L-glutamine, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin. HT-29: McCoy's 5 A modified medium with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin. Cultured in 96 well plates for 24 h.	24 h	MTS	No toxicity	Corrochano et al. 2018
Non-INFOGEST: Perales et al. (2005)	Dilution in DMEM with 10% FBS: 1 in 4 total of blank digesta (ultra pure H_2O + digestion enzymes + salts)	Caco-2	DMEM GlutaMAX™ with 1 g/L glucose, 10% FBS, 1% HEPES, 1% AB, 0.2% fungizone. Cultured in 24 well plates for 24 h.	2 h and 24 h	MTT and trypan blue dye exclusion test	> 85%	Jilani et al. 2020
INFOGEST	Dilution in DMEM with 10% FBS: 1 in 11 total of blank digesta (digestion enzymes + salts) supernatants centrifuged for 5 min at 4000 rpm	Caco-2	DMEM GlutaMAX™ with 4.5 g/L glucose, 10% FBS, 1% HEPES, 1% NEAA, 1% AB. Cultured in 24 well plates for 24 h.	24 h	MTT	No toxicity	De la Fuente et al. 2020
Infant static *in vitro* digestion model: Menard et al. (2018)	Dilution in HBSS: 1 in 10 total of digesta containing protease and lipase inhibitors	Caco-2	DMEM with 4.5 g/L glucose, 10 % FBS, 100 U/mL penicillin, 100 µg/mL streptomycin. Cultured in 96 well plates for 24 h.	4 h	MTT	No toxicity	Bavaro et al. 2021
INFOGEST	Dilution in RPMI-1640 media: to 250 µg protein/cm^2 containing protease inhibitor	Caco-2/ HT-29	Caco-2: MEM with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, 1 mM sodium pyruvate. HT-29: RPMI-1640 with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. Co-culture in 24 well plates for ≥6 day (TEER approx. 200 Ω × cm^2)	2 h	MTT	No toxicity	Corrochano et al. 2019
SHIME	Dilution with HBSS: 1 in 17 total	HT-29	DMEM with 10 % FBS, 2 mM L-glutamine, 1 % NEAA, 1 % antibiotics. Cultured for 24 h.	2 h	Trypan blue exclusion assay	> 80%	Guri, Haratifar, and Corredig 2014
INFOGEST	Heat inactivation at 95^{0}C for 15 min and dilution in DMEM media: 1 in 13 of supernatants centrifuged for 10 min at 5000 g	Caco-2/ HT-29	DMEM with 10% FBS, 1% GlutaMAX™, 1% NEAA, 100 U/mL penicillin and 100 µg/mL streptomycin. Co-culture in 96 well plates for 7 days	24 h	MTT, reactive oxygen and apoptosis assays	No toxicity	Pinho et al. 2021
INFOGEST	Dilution in HBSS: 1 in 3 total of supernatants centrifuged for 10 min at 5000 g	Caco-2	DMEM 1% GlutaMAX™ with 10% FBS, 1% NEAA, 100 U/mL penicillin and 100 µg/mL streptomycin. Cultured in cell culture inserts of 6 well plates for 21-28 days.	2 h	MTT	> 90%	Faria, Melo, and Ferreira 2020
Non-INFOGEST: Perales et al. (2005)	Dilution in DMEM with 10% FBS: 1 in 13.3 total	Caco-2	EMEM with 10% FBS, 1% NEAA, 1% L-glutamine, 1% AB. Cultured for 48h.	4 h daily for 4 consecutive days or 24 h exposure	Trypan blue dye exclusion test	No toxicity	Cilla et al. 2009
INFOGEST model	Dilution in DMEM with 10% FBS: 1 in 11 total	HCT-116 and Caco-2 cells	DMEM with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 5 µg/mL gentamicin. Cultured in 96 well plates.	24 and 48 h	MTT	> 90%	Cilla et al. 2022
Non-INFOGEST: Herman et al. (2007) with modifications (eliminating buffering salts)	Dilution in DMEM: after heat treatment at 100 °C for 10 min and lyophilization	Caco-2	EMEM with 10% FBS Cultured in cell culture PTFE inserts for 20-23 days.	48 h	Neutral red uptake assay	No toxicity	Markell et al. 2017

Abbreviations: AB, antibiotics; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; DMEM, Dulbecco's Modified Eagle Medium; EMEM, Eagles Minimal Essential Media; FBS, Foetal Bovine Serum; HBSS, Hanks Balanced Salt Solution; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; NEAA, Non-essential amino acids; PTFE, polytetrafluoroethylene; RPMI-1640, Roswell Park Memorial Institute medium.

Table 4. Effect of simulated gastric and intestinal fluids on cell monolayer integrity.

Cell-model	Culturing conditions	Exposure time	Test conditions	TEER	Permeability	Reference
Monoculture						
Caco-2	DMEM with 10% FBS, 1% NEAA, AB. Cultured in cell culture PE inserts for 21 days TEER > 1000 $\Omega \times cm^2$	240 min	HBSS (control) Simulated GI fluid (SGIF)	→ SGIF after 4h exposure in HBSS	↑ TJ proteins (occludin, claudin-1, claudin-4, ZO-1, Jam-1 and β-actin)	Bavaro et al. 2021
Caco-2	DMEM with 10% FBS, 1% NEAA, AB, 1% GlutaMAX™ solution TEER > 1000 $\Omega \times cm^2$	120 min	Simulated GI fluid (SGIF)	→ SGIF, <10% reduction after 2h exposure in HBSS		Faria, Melo, and Ferreira 2020
Caco-2	EMEM with 10% FBS Cultured in cell culture PTFE inserts for 20-23 days TEER ≈ 1000 $\Omega \times cm^2$	4/24/48 h	SGIF (non-INFOGEST with pepsin and pancreatin: pH-adjusted, heat-inactivated, frozen, lyophilized and reconstituted in FBS-free media)	↑ SGIF (100-500%), after 48h exposure in FBS-free DMEM	To dextran (4.5 kDa): ↑ SGIF	Markell et al. 2017
Caco-2	RPMI-1640 with 10% FBS, 2 mM L-glutamine, 1% AB. Cultured in HTS cell culture inserts of 24 well plates TEER > 300 $\Omega \times cm^2$	4 h	PBS (control) Digested bread w/o nanoparticles	→ for all samples, after 4h exposure in FBS-free DMEM	↓ actin skeleton and tight junction architecture	Di Silvio et al. 2016
Caco-2	DMEM with 15% FBS, 1% NEAA, 1% AB. Cultured in cell culture inserts for 21-31 days.	120 min	HBSS (control) Fed SIF Fasted SIF	↓ Fasted SIF (75 %) ↓ Fed SIF (98 %)	To lucifer yellow (LY): ↑ Fasted SIF ↑ Fed SIF	Antoine et al. 2015
Caco-2	DMEM with 10% FBS, 1% NEAA; 0.5% antibiotics (AB). Cultured in cell culture PET inserts for 17-21 days. TEER > 300 $\Omega \times cm^2$	120 min.	HBSS (control) Fed simulated intestinal fluid (SIF) Fasted SIF (adjusted to pH 6.5)	→ Fed SIF (↓17.3%^{ns}) → Fasted SIF (↓13.3%^{ns}) after 2h exposure	To FD4: → Fed SIF → Fasted SIF	Gradauer et al. 2015
Caco-2	DMEM with 10% FBS, 1% NEAA, AB. Cultured in cell culture PC inserts for 18-20 days	240 min	HBSS (control) Fed SIF	Vs. T_0: ↓ Fed SIF (> 90%)	To ^{14}C Mannitol: ↑ Fed SIF, ^{3}H-metoprolol ↑ Fed SIF → with mucus	Birch et al. 2018
Caco-2	DMEM with 10% FBS, 1% NEAA, 1% L-glutamine and AB. Cultured in cell culture PE inserts for 21-28 days.	Up to 240 min (TEER) 120 min (Mannitol, ^{3}H-metoprolol)	HBSS (control for permeability) Fasted SIF / Fed SIF Fasted SIF / Fed SIF (modified to use with cell models)	Vs. T_0: → Fasted SIF ↓ Fed SIF (> 80 %, as pH = 5, 635 mOsm/Kg) → Fasted SIF mod → Fed SIF mod	To ^{14}C Mannitol: → Fasted SIF → Fed SIF To ^{3}H-metoprolol: ↓ Fasted SIF ↓ Fed SIF	Patel et al. 2006
Caco-2	DMEM with 10% FBS, 1% NEAA, AB. Cultured in cell culture inserts for 17-18 days. TEER > 150 $\Omega \times cm^2$	120 min	HBSS (control) Fasted SIF FaHIF (fasted human intestinal fluid pool from 11 individuals over 120 min)	Vs. T_0: → Fasted SIF ↓ FaHIF (by 90 %)		Wuyts et al. 2015
Caco-2	DMEM with 10% FBS, 1% NEAA and AB. Cultured in cell culture inserts for 21-25 days. TEER > 250 $\Omega \times cm^2$	60 min	HBSS (control) FaHIF (pool of fasted duodenum fluid from 8 individuals)	↓ FaHIF (> 80%)		Deferme et al. 2003
Caco-2	DMEM with 10% FBS, 1% NEAA, AB. Cultured in cell culture inserts for 21 days	120 min	Simulated GI fluid (SGIF)	→ SGIF		Felice et al. 2018
HT-29MTX	DMEM with 15 % FBS, 1% NEAA, 1% AB. Cultured in cell culture inserts for 21-31 days	120 min	HBSS (control) Fed SIF Fasted SIF	↑ Fasted SIF → Fed SIF, after 2h exposure	To LY: → Fasted SIF → Fed SIF	Antoine et al. 2015
HT-29	DMEM with 10% FBS, 1% NEAA, AB. Cultured in cell culture PC inserts for 25-27 days	240 min	HBSS (control) Fed SIF	Vs. T_0: ↓ Fed SIF (> 55%)	^{14}C Mannitol: ↑ Fed SIF, ^{3}H-metoprolol: → Fed SIF	Birch et al. 2018
Co-culture						
Caco-2/HT-29MTX Ratio 1:2	DMEM with 15 % FBS, 1% NEAA, 1% AB. Cultured in cell culture inserts for 21-31 days	120 min	HBSS (control) Fed SIF Fasted SIF	↓ Fed SIF ↓ Fasted SIF, after 2h exposure	To LY: ↑ Fasted SIF → Fed SIF To LDH: → Fed SIF apic ↑ Fed SIF apic-baso	Antoine et al. 2015

Table 4. Continued.

Cell-model	Culturing conditions	Exposure time	Test conditions	TEER	Permeability	Reference
Caco-2/HT-29 Ratio 1:1	DMEM with 10% FBS, 1% NEAA, AB. Cultured in cell culture PC inserts for 25-27 days	240 min	HBSS (control) Fed SIF	Vs. T_0: ↓ Fed SIF (> 65%)	^{14}C Mannitol: ↑ Fed SIF, ^{3}H-metoprolol: → Fed SIF	Birch et al. 2018

Abbreviations: AB, antibiotics; DMEM, Dulbecco's Modified Eagle Medium; EMEM, Eagles Minimal Essential Media; FBS, Foetal Bovine Serum; FD4, Flourescein isothiocyanate-dextran; GI, gastrointestinal; HBSS, Hanks Balanced Salt Solution; HTS, high throughput screening; JAM-1, junction adhesion molecule-1; Mod; modified; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide; NEAA, Non-essential amino acids; PBS, Phosphate Buffered Saline; PC, polycarbonate; PE, Polyethylene; PET, Polyethylene terephthalate; PTFE, polytetrafluoroethylene; RPMI-1640, Roswell Park Memorial Institute medium; SGIF, simulated gastrointestinal fluids; SIF, simulated intestinal fluid; TEER, transepithelial electrical resistance; TJ, tight junction protein; ZO-1, Zonula occludens-1.

Table 5. Recommendations for coupling *in vitro* food digestion with *in vitro* epithelial absorption.

When?	What?	Why?
Before GI digestion	Include a digesta control (i.e., no-food control)	To monitor toxicity of digestive enzymes and bile salts on cells
At the end of GI digestion	If using Pefabloc SC, use 0.5 mM	To reduce cytotoxicity without compromising digestion termination
	Check osmolality and adjust to 290-310 mOsm/kg	To ensure digesta is suitable for cell studies
	Check pH and adjust to 6.2-8	To ensure digesta is suitable for cell studies
	Dilute to final bile salts concentration of ~1mM	To ensure digesta is suitable for cell studies
Before absorption studies	Filter sterilize using 0.45 µm or 0.22 µm filters	To ensure sterility in cell studies
	Perform cell health check with food digesta	To ensure food digesta will not detach or damage monolayers; to assist inter-lab comparisons
During absorption studies	Measure TEER values at time zero and at the end of absorption experiment	To monitor monolayer damage over the experiment
At reporting	State if an enzyme inhibitor is used (type and concentration)	To assist inter-lab comparisons
	Describe in detail detoxification steps employed	To assist inter-lab comparisons
	Report raw data of cell health check using digesta control compared to media	To assist inter-lab comparisons
	Report raw data for digesta control compared to vehicle control	To assist inter-lab comparisons

Abbreviation: TEER, transepithelial electrical resistance.

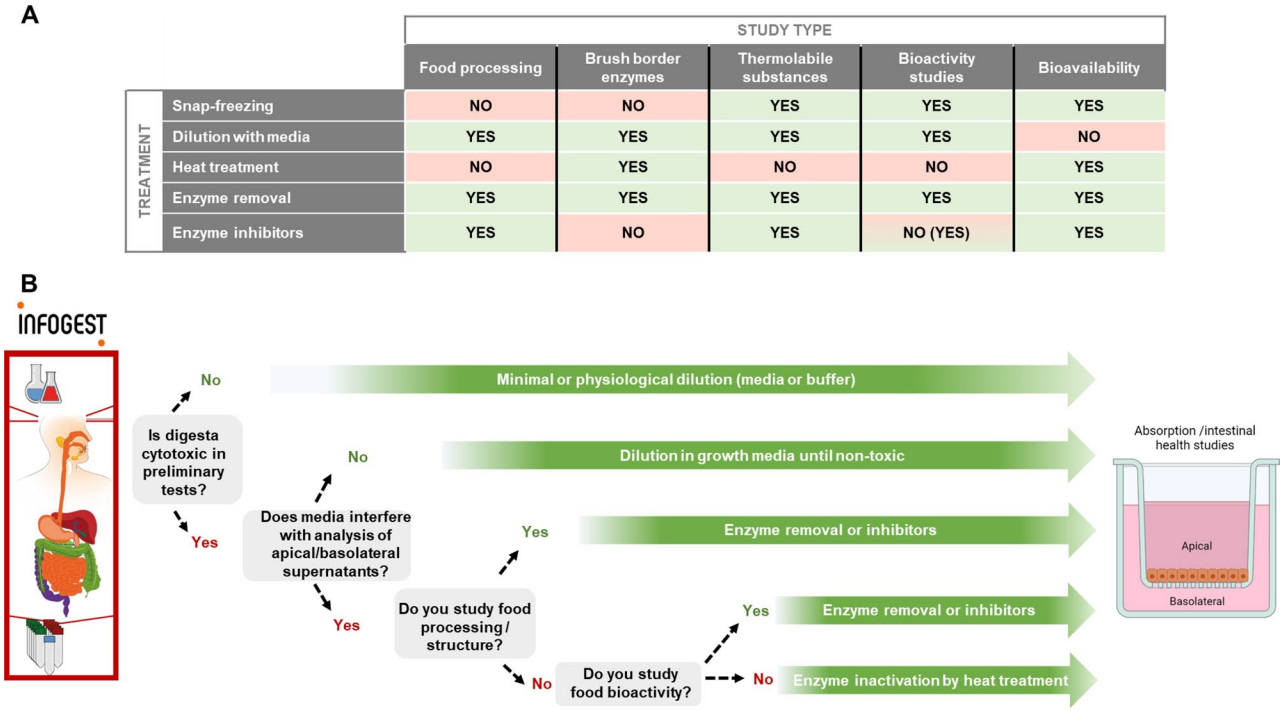

Figure 1. Flowchart to select suitable detoxification steps.

here will translate to Caco-2 polarized monolayers cultured in membrane inserts. Corrochano et al. (2018) employed MTS assay and tracked redox markers to assess biocompatibility of food digesta. MTS values in either undifferentiated HT-29 or Caco-2 cells seeded in 96 well formats were similar regardless of whether cells were treated with HBSS buffer or digesta control. In addition, HT-29 cells were not oxidatively stressed with this digesta control (Corrochano et al. 2018). In Caco-2 cells, activities of individual redox enzymes were also unaffected by digesta control, although there were some differences in mRNA transcript levels of redox markers (Corrochano et al. 2018). Jilani et al. (2020) tracked reactive oxygen species and cell cycle progression in undifferentiated Caco-2 cells treated with NI blank digesta. Although this digesta control significantly reduced Caco-2 mitochondrial membrane potential and altered Caco-2 sub-G1 cell phase distribution after 2 h compared to media control, these differences were transient and not observed at 24 h (Jilani et al. 2020). De la Fuente et al. (2020) employed MTT, cell cycle distribution, oxidative stress, mitochondrial membrane potential and intracellular calcium assays in undifferentiated Caco-2 cells to evaluate biocompatibility of GI digesta. The diluted digesta control with a pH of 7.5 and 296 mOsm/L displayed no cytotoxic effects. Bavaro et al. (2021) utilized MTT to establish that undifferentiated Caco-2 cells in 96 well format treated for 4 h with control digesta had similar viability to cells treated with HBSS buffer alone. This blank digesta was generated from an INFOGEST method modeling the infant gut (Menard et al. 2018). Guri, Haratifar, and Corredig (2014) selected a 1 in 17 dilution of digesta for Caco-2/HT-29MTX monolayer absorption studies based on data generated from Trypan blue viability assays in undifferentiated HT-29 cells.

Some studies employ monolayers differentiated for 7-10 days for the initial viability screen. Corrochano et al. (2019) allowed Caco-2/HT-29 co-cultures to differentiate for 10 days prior to MTT assays. Results guided the selection of digesta at 250 µg protein/cm^2 for subsequent TEER assays and permeability assays (Corrochano et al. 2019). Pinho et al. 2021 treated 7 day old Caco-2/HT-29 monolayers in a 96 well format with digested milk. Post GI digestion, the sample was heat inactivated and diluted in DMEM. Monolayers treated for 24 h remained healthy and similar to media alone as determined by MTT, reactive oxygen species and apoptosis assays (Pinho et al. 2021).

Once researchers are confident in their detoxification steps, it is then possible to proceed to absorption studies. Table 4 details the impact of digestive fluids on Caco-2, HT-29 and Caco-2/HT-29MTX monolayers. These studies routinely report on TEER values before and after treatment to monitor barrier integrity. We recommend that TEER values are also reported for both digesta control and vehicle control at both time points.

Bavaro et al. (2021) observed that digesta control did not significantly alter TEER values nor levels of tight junctions proteins in Caco-2 monolayers, compared to HBSS buffer over a 4 h period. Faria, Melo, and Ferreira (2020) allowed for a maximum 10% reduction in TEER in response to digesta in HBSS over 2 h. Markell et al. (2017) also treated Caco-2 monolayers with NI-GI gastric and intestinal controls but diluted in FBS free media. There was no difference in TEER values, or permeability (uptake of dextran or neutral red) for gastric control samples after 48 h compared to media control, but GI control led to significantly higher TEER values (Markell et al. 2017). Di Silvio et al. (2016) did observe alterations by laser scanning confocal microscopy of the Caco-2 actin skeleton and tight junction architecture with GI digesta samples diluted in FBS free media over 4 h. These adjustments did not alter TEER values (Di Silvio et al. 2016).

Antoine et al. (2015) reported on the viability of Caco-2, HT-29MTX or Caco-2/HT-29MTX monolayers in the presence of fed and fasted intestinal fluids (without digestive enzymes) and provided MTT, lactate dehydrogenase and permeability data. Caco-2 TEER values fell dramatically after 2 h incubation with fed and fasted fluids but co-cultures and HT-29MTX were more robust with minimum reductions and noticeable recovery (Antoine et al. 2015). The apical mucus barrier of HT-29MTX appears to add a protective layer (Antoine et al. 2015). This protective effect of mucus was confirmed by others (Birch et al. 2018; Wuyts et al. 2015) who applied a synthetic mucus to Caco-2 monolayers prior to treatment with either human duodenal aspirates (Wuyts et al. 2015) or artificial intestinal fluid (Birch et al. 2018). Where mucus is absent, TEER values in Caco-2 monolayers treated with fasted and fed intestinal fluids fell by a minimum of 11-17% respectively over 2 h with corresponding significant increases in permeability (Gradauer et al. 2015).

Although many different cell health parameters can be assessed at least one must be performed prior to monolayer treatment with digesta. For monolayer experiments, epithelial integrity checks pre and post treatment with digesta are essential. Recording TEER remains a reliable, quick and noninvasive method to evaluate monolayer disruption. We recommend reporting raw data where possible rather than normalized data.

Conclusions and recommendations

It is important to note that the choice of detoxification steps selected will depend on the food component or foods of interest and the purpose of the study. However currently there is no 'one size fits all' protocol. All our recommendations are summarized in Table 5. Figure 1 outlines a flowchart to assist researchers in the selection process. Each step has its own advantages and limitations. Dilution with media appears to be the least destructive. However, post adsorption quantification analysis of the compound of interest can then be a challenge. On the other hand, heat inactivation of digestive enzymes is effective but cannot be used in studies where the food compound of interest is thermolabile or where the effect of food processing, food structure or delivery of bioactive components is under investigation. In such studies the use of enzyme inhibitors may be a better choice. However, the use of inhibitors is likely to interfere with Caco-2 brush border enzyme activities. A 1 in 10 dilution in buffer will dilute bile salts and enzyme inhibitors, and can be used to adjust osmolarity, if required. In

conclusion, reaching a consensus protocol may not be possible but agreeing a common detoxification workflow is possible.

It is important to note that achieving biocompatiblity with Caco-2 does not automatically translate to all cell lines, so health checks must be performed on cells of interest. In our ultimate collective aim for a consensus protocol in the future, our recommendations encourage researchers to (1) describe in detail the steps taken to detoxify (2) include a digesta control in all experiments and (3) report on cell health checks pre and post digesta treatment (Table 5).

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Abbreviations

DMEM	Dulbecco's Modified Eagle Medium
EMEM	Eagles Minimal Essential Media Fasted SIF, Fasted simulated intestinal fluids without digestive enzymes
Fed SIF	Fed simulated intestinal fluids without digestive enzymes
FBS	Fetal Bovine Serum
GI	gastrointestinal
HBSS	Hanks Balanced Salt Solution
MEM	Minimal Essential Medium
MWCO	Molecular weight cutoffs
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide
NEAA	Non-essential amino acids
NI-GI digestion	not INFOGEST gastrointestinal digestion
PTFE	polytetrafluoroethylene
RPMI-1640	Roswell Park Memorial Institute medium
SGIF	simulated gastrointestinal fluids
TEER	transepithelial electrical resistance

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