Invited review: Dairy proteins and bioactive peptides: Modeling digestion and the intestinal barrier

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ABSTRACT

Dairy products are one of the most important sources of biologically active proteins and peptides. The health-promoting functions of these peptides are related to their primary structure, which depends on the parent protein composition. A crucial issue in this field is the demonstration of a cause-effect relationship from the ingested protein form to the bioactive form *in vivo*. Intervention studies represent the gold standard in nutritional research; however, attention has increasingly been focused on the development of sophisticated *in vitro* models of digestion to elucidate the mechanism of action of dairy nutrients in a mechanistic way and significantly reduce the number of *in vivo* trials. On the other hand, the epithelial intestinal barrier is the first gate that actively interacts with digestion metabolites, making the intestinal cells the first target tissue of dairy nutrients and respective metabolites. An evolution of the *in vitro* digestion approach in the study of dairy proteins and derived bioactive compounds is the setup of combined *in vitro* digestion and cell culture models taking into consideration the endpoint to measure the target organism (e.g., animal, human) and the key concepts of bioaccessibility, bioavailability, and bioactivity. This review discusses the relevance and challenges of modeling digestion and the intestinal barrier, focusing on the implications for the modeling of dairy protein digestion for bioactivity evaluation.

Key words: dairy protein, bioactive peptide, *in vitro* digestion, intestinal cell

INTRODUCTION

Dairy products are one of the most important sources of high-value proteins and bioactive peptides. Beyond their well-known nutritional values, they exhibit an extensive range of physiological effects that promote general health through their beneficial effects on spe-

cific organs and tissues (Meisel and FitzGerald, 2003; Martínez-Maqueda et al., 2012a). The nutraceutical industry has developed a strong interest in studying and discovering bioactive molecules from complex food mixtures, such as dairy products, for the production of health-promoting functional foods. The most important health benefit of dairy proteins is obtained following gastrointestinal digestion and peptide release (Petrat-Melin et al., 2015; Giromini et al., 2017). A peptidome study performed on human jejunal effluents after ingestion of milk proteins identified protein domains resistant to gastrointestinal digestion (Sanchón et al., 2018). Some of these resistant peptide fragments correspond to previously described sequences that might exert their physiological function by directly interacting with the intestinal epithelium or by being absorbed into the bloodstream and distributed throughout the body, where they elicit unique functions (Shimizu, 2004). To evaluate the health-promoting role of these bioactive peptides, attention has increasingly been focused on *in vitro* digestion models. Although intervention studies represent the gold standard in nutritional research, *in vitro* methods for the simulation of the gastrointestinal protein digestion process are currently widely used in food and nutraceutical research (Minekus et al., 2014; Guerra et al., 2012). These methods are rapid, less expensive, and involve fewer ethical restrictions compared with *in vivo* trials; moreover, they can elucidate the mode of action of nutrients in a mechanistic way with a significant reduction of the use of laboratory animals, following the principle of replacement, reduction, and refinement of animal studies. Moreover, the attempt of modeling milk proteins digestion *in vitro* can determine the bioaccessibility of dairy peptides, the effect of their chemical form, and the synergistic effect of all the dairy peptides produced during the digestion process (Guerra et al., 2012). The epithelial barrier is the first gate, which actively interacts with digestion components, making the intestinal cells one of the first target tissues of dairy proteins and derived peptides. Intestinal cell-based models have been used in food and feed research as predictive tools to study the absorption and the bioactivity of dairy proteins and derived pep-

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tides (Contreras et al., 2011). The setup of combined in vitro digestion and cell culture models represents an evolution of the in vitro digestion approach currently applied to study dairy nutrients and derived peptides bioactivity. This review discusses the relevance and challenges in modeling digestion and intestinal barrier function, focusing on the implications for modeling of dairy protein digestion for bioactivity evaluation.

BIOACTIVE COMPOUNDS OF MILK AND DAIRY PRODUCTS

Milk and dairy products represent complete food sources of macro- and micronutrients that also impart a wide range of health benefits to the consumer due to the amount of biologically active molecules they possess (Hill and Newburg, 2015). Among dairy products, cheese whey is a sustainable and high-value by-product of the dairy processing industry (Smithers, 2015), characterized by a high amount of proteins and oligosaccharides with an elevated potential for the production of functional foods. The bioactivity of dairy compounds is largely derived from high-quality proteins and peptides (Baldi et al., 2005; Politis and Chronopoulou, 2008; Fox et al., 2015) and partially derived from milk lipids (Haug et al., 2007; Baldi and Pinotti, 2008; Rosetta and Baldi, 2008), oligosaccharides (Boudry et al., 2017; Douëllou et al., 2017), and micronutrients such as calcium, phosphorus, sodium, and vitamins (Caroli et al., 2011). Among the wide range of dairy bioactive molecules, proteins and peptides have been largely investigated (Martínez-Maqueda et al., 2012b; Petrati-Melin et al., 2015; Giromini et al., 2017) and their bioactivity has been extensively reviewed in recent years (Purup and Nielsen, 2012; Holt et al., 2013; Sultan et al., 2018; Table 1).

In spite of the vast number of studies focused on the evaluation of milk proteins and peptide functionality, an imbalance exists in the literature, as most of this attention has focused on the effect of specific peptides (e.g., β -casomorphin, lactoferricin) with a clear evidence gap

regarding the synergistic effect of the peptide milieu from casein and whey proteins (Fekete et al., 2016a; Giromini et al., 2017), particularly at the gastrointestinal level. Nevertheless, in vivo studies investigating the bioactive properties of dairy proteins are still rare and sometimes produce controversial results (Egger and Ménard, 2017). Based on this information, studying the effects of milk and dairy bioactive peptides as a whole and considering the natural co-occurrence of bioactive peptide in dairy products is of paramount importance.

IN VITRO MODELS OF DAIRY PROTEIN DIGESTION

The study of dairy protein digestion represents a prerequisite for the determination of the physiological properties of encrypted peptides. When in vitro models mimicking the physiology of protein digestion are designed, the key concepts of bioaccessibility, bioavailability, and bioactivity must be studied. The fraction of ingested protein that is effectively released from the food matrix and available for intestinal absorption is defined as bioaccessible, whereas bioavailability is defined as the quantity of ingested protein that becomes available for utilization at the blood vessel level (Parada and Aguilera, 2007; Figure 1).

The bioaccessibility of a protein is a key parameter for the determination of its bioavailability. Thus, bioavailability is the result of 3 main steps: protein digestibility or solubility in the gastrointestinal tract, absorption by the intestinal epithelial cells, and tissue distribution. Bioavailability is usually determined by in vivo assays, which consider individual variability, physiological state, dose, and the co-presence of other food components. Thus, although bioavailability and bioaccessibility are often used indistinctly, they are not equivalent; it must be clarified that bioavailability includes the concept of bioactivity (Etcheverry et al., 2012). Bioactivity represents all events linked to how the bioactive compound reaches the target tissue, interacts with biomolecules, and how it is metabolized and transformed to induce a physiologic response. The

Table 1. Selection of comprehensive reviews on dairy bioactive proteins and peptides for further reading

Target district	Bioactive effect	References
Gastrointestinal system	Antimicrobial, barrier function, mucus production	Akalin, 2014; Martínez-Augustin et al., 2014; Boutrou et al., 2015; Miralles et al., 2018a; Sultan et al., 2018
Cardiovascular system	Antihypertensive, blood pressure regulation	Aluko, 2015; Fekete et al., 2013, 2015; Beltrán-Barrientos et al., 2016; Bhat et al., 2017; Egger and Ménard, 2017; Miralles et al., 2018a
Immune system	Immunomodulatory, cytomodulatory	Santiago-López et al., 2016; Egger and Ménard, 2017; Cicero et al., 2017; Miralles et al., 2018a
Nervous system	Opioid effect, cognitive function	Visioli and Strata, 2014; Nongonierma and FitzGerald, 2015, 2016
Metabolic health	Insulin control, hypocholesterolemic, glucose control	Nongonierma and FitzGerald, 2015; Fekete et al., 2016b; Egger and Ménard, 2017; Miralles et al., 2018a
Diverse	Antioxidant, mineral binding	Nongonierma and FitzGerald, 2015; Sultan et al., 2018

Figure 1. Bioaccessibility and bioavailability concepts applied to dairy proteins (adapted with permission from Guerra et al., 2012).

bioavailability and bioactivity of a peptide are dependent on gastrointestinal parameters, food processing, and food matrix complexity, which, altogether, affect the efficiency of the digestion and absorption processes. Claims of what a dairy food can do (health properties or disease reduction) are based on bioactivity studies. The development and validation of in vitro models for bioaccessibility, bioavailability, and bioactivity studies are a difficult task and should consider the complexity of the gastrointestinal tract, gut ecosystem, and the physiology of target subject.

In vivo protein digestion begins when food proteins reach the stomach, and digestion is assisted by the highly acidic gastric environment. The gastric digestion conditions have a pH ranging from 1 to 5 and a transit time of 30 min to 3 h. In the stomach, the gastric juice primarily promotes protein digestion. This acidic environment in the stomach causes the unfolding of proteins, and the gastric enzymes are thus granted easier access to the protein structures to cleave them

into smaller pieces. At the gastric level, casein proteins are extensively degraded by pepsin hydrolysis in both adults and infants (Boland et al., 2014). The globular structure of whey proteins renders them more resistant to gastrointestinal hydrolysis. In particular, β -LG can interact with phosphatidylcholine of the gastric mucosa while not being affected by gastric digestion, which, in turn, protects the protein from duodenal digestion. Lactoferrin, although relatively resistant to digestion by proteases in the gastrointestinal tract, can be actively hydrolyzed during gastric digestion into the most potent bioactive peptide, lactoferricin (Baldi et al., 2005; Furlund et al., 2013). Diverse results have been reported on the digestibility of the second major whey protein, α -LA (Inglingstad et al., 2010; Nik et al., 2010). Even though casein proteins have a structure that makes them sensitive to enzymatic hydrolysis, they are considered slow proteins due to the longer retention in the stomach compared with whey protein (fast protein). The longer stomach retention of caseins

lead to a longer persistence of casein-derived AA in plasma compared with whey-derived AA (Boirie et al., 1997).

Gastric-protein digestion in the small intestine, the longest section (Guerra et al., 2012) of the digestive tube, representing the first highly selective communication barrier between the gastrointestinal environment and the metabolic organs. Trypsin and additional enzymes, such as carboxypeptidase and aminopeptidase, are present in the small intestine and contribute to the intestinal hydrolysis of proteins. Di- and tripeptides of the digestate can be transported by specific transporters or hydrolyzed into AA at the intestinal epithelial cell level and subsequently absorbed into the bloodstream and distributed throughout the body, where they elicit unique functions (Shimizu, 2004). Unabsorbed polypeptides reach the large intestine (cecum, colon, and rectum) where fermentation and water reabsorption occur with a transit time over 12 h in adults (Guerra et al., 2012).

Static Models to Study Dairy Bioactives

The design of a model has to take into account several aspects related to the food matrix, composition, and human and animal physiology. Static and dynamic models with single or multicompartmental systems have been developed to simulate the physiological process of digestion. Static models consist of a 2- or 3-step procedure simulating the digestive processes in the mouth, stomach, and small intestine, with the addition of simulated gastric and intestinal juices and incubation at 37°C for a time relevant to the specific

compartment (Minekus et al., 2014). Static models are relatively cheap and the most widespread among researchers studying digestion and the bioactivity of various nutrients, including milk and dairy proteins. These models reproduce the digestion phases at a fixed temperature and pH. The oral, gastric, and intestinal phases are simulated in the same system using single enzymes (pepsin), enzyme combination (pancreatic and liver enzymes), or a defined medium that reproduces gastric or intestinal juices. Some of the static models use mechanical force to simulate peristaltic movements and may include mechanisms for undigested food or end-product removal (Guerra et al., 2012). By 2014, hundreds of static systems had been developed and used to study digestion, making the standardization of the results and their reproducibility a difficult task. The first attempt to validate an international consensus protocol was performed by Minekus et al. (2014). Those authors developed a harmonized protocol, improving health properties of food by sharing our knowledge on the digestive process (**INFOGEST**), to simulate the in vitro digestion of food to increase the reproducibility of results among different laboratories and countries (Figure 2). The INFOGEST protocol was set up and validated on milk powder samples, and the bioaccessibility of milk proteins has been used to assess the suitability of the protocol.

Compared with other in-house methods, the INFOGEST protocol revealed a high hydrolysis potential and a more homogeneous protein pattern in the digestion samples with no intact proteins detectable (Bohn et al., 2017). In general, the widespread application of the INFOGEST protocol is currently improving the com-

Figure 2. The static in vitro digestion protocol developed in the frame of the COST Action INFOGEST (Minekus et al., 2014). The protocol allows the standardization of the oral, gastric, and intestinal digestion conditions in vitro.

parability of the results among different laboratories studying milk protein hydrolysis or bioactivity. Egger et al. (2016) studied the fate of casein and whey proteins after ingestion using the INFOGEST protocol and found that casein is mainly hydrolyzed after the pepsin phase, whereas whey proteins, such as β -LG, were resistant to pepsin attack. Furthermore, comparison of the INFOGEST protocol with in vivo models was first performed in a pig model and, later, in humans. The same sample of milk powder used to validate the INFOGEST was tested in a swine trial, showing that the in vitro gastric and intestinal phases reflected in vivo milk protein hydrolysis and bioaccessibility (Bohn et al., 2017). In a recent study, Sanchón et al. (2018) performed a comparison of the INFOGEST protocol with human digestion data and found that it resembled the in vivo intestinal digestion and peptide release. After the harmonization of the static digestion protocol, several studies have been performed using the INFOGEST protocol to study the bioaccessibility and the bioactivity of milk and dairy proteins and peptides. The digestion, hydrolysis, and peptide profile from casein was investigated by Asledottir et al. (2017, 2018) in digestates from casein and from ex vivo gastrointestinal digestion. In our research group, we digested a selection of whey, casein, and plant proteins in vitro and found an increase in angiotensin I-converting enzyme (**ACE**) inhibitory activity after in vitro dairy protein digestion (Fekete et al., 2016a; Giromini et al., 2017). The bioactivity detected in vitro was substantiated by 2 human intervention studies where casein and whey protein were administered in powder form orally to human volunteers (Fekete et al. 2016a, 2018). We found that whey and casein protein administration resulted in a lower blood pressure and improved cardiovascular biomarkers in adults with prehypertension and mild hypertension. Furthermore, De Noni et al. (2015) and Cattaneo et al. (2017) studied the effect of the INFOGEST protocol on Grana Padano cheese digesta and the casein profile. The INFOGEST protocol was also used to study the effect of a lipoprotein matrix structure on the digestion kinetics of whey proteins (Mat et al., 2016). This evidence demonstrates the suitability of the protocol to study milk protein digestion with a high predictive power on the released peptides and bioaccessibility. In turn, this can be used to investigate the associated bioactivity. In general, static models are particularly useful for the standardization of results, providing an enormous boost to the practical and economic potential of bioactivity measurements. Nevertheless, despite their potential and broad applicability to study bioaccessibility (and bioactivity), none of the static models reproduce the dynamic environment of the intestine.

Dynamic Models to Study Dairy Bioactives

Digestion is a dynamic process. Among the dynamic models available, either mono- or multicompartmental systems have been used for the study of dairy protein digestion. In the dynamic models, the exact conditions within the different compartments change over time to simulate the in vivo digestion processes. Dynamic digestion models account for the different digestive secretions, which are added to the compartments of the model at specific time points. This addition can either follow a stable secretion rate (as for the simulated gastric juice in the human gastric simulator), it can follow a preprogrammed pattern (allowing the rate to change over time; e.g., in the TIM-1 model), or it can be programmed to change in response to other parameters, such as the volume of the model (e.g., gastric secretion in the dynamic gastric model; Thuenemann et al., 2015). Concerning dairy protein digestion evaluation, dynamic models have only been applied in few cases. A summary of the main application of dynamic models to study dairy protein digestion and or bioactivity is reported in Table 2.

Specific dynamic digestion models tailored for specific human populations have also been studied (Shani-Levi et al., 2017). The gastrointestinal system and the digestion process of an adult differs from that of a neonate or a small child, making the standardization of the digestion protocols difficult to accomplish. These differences need to be considered when choosing or developing a digestion protocol. Most of the established in vitro models have been designed to simulate the specific processes taking place within an adult human. The digestion process differs in children, seniors, and pathological patients (e.g., inflammatory bowel disease); therefore, the in vitro models developed to simulate the digestion in adults will not accurately reflect processes occurring in those individuals (Kamstrup et al., 2017). Customized models may represent new tools to accurately evaluate the bioaccessibility of tailored and personalized foods for a specific stratum of the human population, such as infants, the elderly, and patients with gastrointestinal disorders. In the near future, dynamic digestion systems will likely become unavoidable for understanding the mechanisms of digestion and absorption, especially because of the increased ethical and economic constraints of in vivo trials. Therefore, the harmonization of a dynamic digestion protocol represents one future goal to achieve in nutritional research.

Egger et al. (2017) took a step forward in the validation of a dynamic model. In their study, dairy protein was digested with the INFOGEST protocol and the

Table 2. Dynamic model complexity and application to milk and dairy digestion

Dynamic model	Origin/location	Complexity	Application	Reference
Dynamic gastric model (DGM)	Institute of Food Research (Norwich, UK)	Mono-compartmental (gastric digestion)	Milk protein digestion — proteomic approach	Zhang et al., 2014
Human gastric simulator (HGS)	University of California (Davis)	Mono-compartmental (gastric digestion)	Whey protein hydrolysis	Guo et al., 2014
TIM-1	TNO (the Netherlands)	Multicompartmental (gastric and small-intestinal digestion)	Digestion of whey protein extract	Nabil et al., 2011
Simulator of the gastrointestinal tract (Simgi)	Institute of Food Science Research CIAL (CSIC-UAM, Madrid, Spain)	Multicompartmental (stomach, small intestinal and colonic microbiota)	Digestion of whey protein concentrate	Miralles et al., 2018b
DIDGI system	INRA (Paris, France)	Multicompartmental (gastric and small intestinal digestion)	Peptide mapping Milk proteolysis Intestinal bioaccessibility of AA	Sánchez-Rivera et al., 2015; de Oliveira et al., 2016a,b
Simulator of the human intestinal microbial ecosystem (SHIME)	Ghent University (Ghent, Belgium)	Multicompartmental (gastric, small and large intestinal digestion)	Whey retentate effect on the microbial community	Meddah et al., 2001

dynamic protocol DIDGI (Ménard et al., 2015). The hydrolysis of proteins was compared between the 2 systems, as well as to in vivo porcine digestion. The gastric and intestinal endpoints of both in vitro systems showed good reproducibility compared with porcine in vivo digestion. In particular, during the gastric phase, the kinetic of protein hydrolysis in DIDGI showed a dynamic evolution and was closer to the in vivo situation.

Generally, dynamic models to study dairy protein digestion and the bioaccessibility of AA are capable of mimicking the gastric digestion of milk proteins with a high predictive power (Miralles et al., 2018b). However, to date, the lack of standardization and the high cost of the dynamic models limits their widespread application.

IN VITRO MODELS OF THE INTESTINAL BARRIER

The intestine is a composite environment responsible for processing and absorbing nutrients also serving as a selective epithelial barrier to protect against pathogens entering with food (Robert et al., 2017). The intestinal epithelial barrier consists of different types of cells, including the absorptive enterocytes and other specialized intestinal epithelial cells (IEC; Peterson and Artis, 2014; Table 3). These cells cooperatively work together to maintain the dynamic barrier between the luminal and outer environments to avoid the hazards associated with the intrusion of undesired substances, such as pathogenic or opportunistic bacteria, viruses, food derived antinutritional compounds, and mycotoxins.

In addition to these major cell types, microfold, cup, tuft, and M cells can also be found in the gastrointestinal epithelium (Gerbe et al., 2012). Enterocytes and colonocytes are the main representative cells in the small and large intestine, respectively, as they comprise 80% of the IEC (Goto and Kiyono, 2012). Enterocytes and colonocytes are specialized in the absorption of nutrients (e.g., peptides) in the small and large intestines, respectively. Peptide absorption occurs through different pathways, including paracellular diffusion via tight junction, passive diffusion via enterocytes or endocytosis, and carrier-mediated diffusion via the PepT1 H⁺/peptide cotransporter (PepT1). Wang and Li (2017) reported that peptides transported by PepT1 showed higher bioavailability than peptides transported through the paracellular route. An alternative absorption pathway, suggested by Martínez-Maqueda et al. (2012a), involves the activity of small peptides on opioid receptors present in the gut wall. The absorption step represents a key point in dairy peptide bioactivity at the target tissue.

Di- and tripeptides and AA from ingested proteins are easily absorbed by intestinal epithelial cells; however,

Table 3. Major intestinal epithelial cell types and their role in the intestinal environment (Furness et al., 2013; Johansson et al., 2013; Peterson and Artis, 2014)

Major epithelial cells	Role in the gastrointestinal system	Gastrointestinal site
Enterocytes/colonocytes	Nutrient absorption Production of AMPs- C-type lectin regenerating islet-derived protein III γ (REGIII γ)	Villus
Goblet	Secretion of mucins, trefoil factor (TFF) and resistin-like molecule- β (RELM β)	Crypt undifferentiated/villus after differentiation
Enteroendocrine	Digestion Hormones secretion as a response to the presence of nutrients	Crypt undifferentiated/villus after differentiation
Paneth	Mucosal host defense/secretion of antimicrobial peptides (AMPs)	Crypt bottom

orally administered long-chain bioactive peptides may have complications in crossing the intestinal epithelial cell barrier and therefore lose the ability to act *in vivo*. Taking oral administration of ACE inhibitory peptides as an example, these peptides must remain intact and active after intestinal transport and contact with brush border peptidases. The *in vivo* ACE inhibitory effect of dairy proteins (Fekete et al., 2018), in fact, is mediated through receptors on the intestinal wall (Miner-Williams et al., 2014; Sangsawad et al., 2018).

In an *in vitro* study performed with Caco-2 cells, Satake et al. (2002) demonstrated that lactotripeptides are effectively transported across the epithelium via the paracellular route and showed activity both *in vitro* and *in vivo*. However, Vij et al. (2016) indicated that the major route of VLPVPQK casein peptide transport might be via the PepT1 transporter rather than via transcytosis or a paracellular route. Based on this, it is of paramount importance to elucidate the transport permeability and route of dairy peptides across the intestinal epithelium to correctly predict their bioactivity potential.

Among IEC, goblet cells are the second most abundant after enterocytes; they have a crucial role in producing the mucus layer which covers the intestinal mucosa. The mucus layer is an integral structural component of the intestinal barrier that acts as a medium for the protection and lubrication of the epithelium as well as transport between the luminal content and the epithelial lining (Deplancke and Gaskins, 2001). Several studies have been focused on the effect of dairy proteins in mucin production and mucus gene expression, and the beneficial role of dairy peptides have been intensively studied in mucus-producing goblet intestinal cell (Martínez-Maqueda et al., 2013a; Plaisancié et al., 2013). The mucus layer is maintained through a continuous secretion of mucins by goblet cells. Mucins consist of a core protein structure linked to numerous hygroscopic and hydrophilic oligosaccharide side-chains that contribute to the formation of a gel-like structure and can be divided into secreted and membrane-associated

mucins (Liévin-Le Moal and Servin, 2006; Lang et al., 2007; Figure 3).

The epithelial intestinal barrier is the first gate, which actively interacts with dairy proteins after ingestion, making the intestinal cells one of the first target tissues for the bioactive peptides. Intestinal cell-based models have been widely used in nutritional research as a predictive tool to study the absorption, mechanism of action, and different nutritional and bioactive effects of food compounds, including milk and dairy proteins, at the gastrointestinal level. Intestinal epithelial cells are strongly influenced by interactions with neighboring cells (e.g., immune cells); therefore, the co-cultivation of intestinal epithelial cells with immune cells allow investigation of the effects of bioactive proteins and peptides on intercellular communication. Intestinal or immune co-cultures, however, have not been used widely due to the lack of standardization in immune cells response,

Figure 3. Graphical representation of membrane-bound glycoproteins adhered to the goblet epithelial wall (MUC1, MUC3, MUC4, MUC12, MUC13, MUC16, MUC17, and MUC20) and secreted in the mucus [MUC2 (most abundant in the small intestine), MUC5AC, MUC5B, and MUC6].

which depends on the donor and can lead to increased variability between experiments (Kleiveland, 2015).

The choice of the appropriate cell model is essential for the outcome when dairy bioactives are tested in cell culture (Purup and Nielsen, 2012). The cell model should be a compromise between easy handling and high human or animal predictive power, providing data that supports the interpretation of results for an in vivo situation. Primary cells are rarely used in nutritional research due to scarce reproducibility of the results and short life span. Indeed, primary cells survive only few days in cell culture, making the experimental reproducibility a major limit (Cencič and Langerholc, 2010; Langerholc et al., 2011). Among the cell lines available, Caco-2 cells have been widely used for absorptive, proliferative, and mechanistic studies; they express the characteristics and functionality of enterocytes upon reaching confluence (Langerholc et al., 2011). The human colon carcinoma HT-29 cells, besides being a model to study the different aspects of the biology of human cancer, received interest in food digestion and bioactivity studies. Goblet cells have the crucial role of secreting mucins (Goto and Kiyono, 2012) and can be mimicked in vitro using mucus-secreting cell line HT29-MTX-E12. The HT29-MTX-E12 cells were extensively used to study the role of bioactive dairy peptides in mucus production and gut functionality (Martínez-Maqueda et al., 2013b; Giromini et al., 2015). A summary of the major intestinal cell lines used to study dairy protein and peptide absorption and bioactivity is reported in Table 4. The literature of in vitro dairy protein studies shows that the Caco-2 cell line is mainly used in absorption or transport studies, whereas the HT29-MTX cell line is mainly involved in mucus-modulation studies. Both IPEC-J2 and IPEC-I cell lines are good candidate models to study antibacterial peptides at the gastrointestinal level (Mariani et al., 2009; Brosnahan and Brown, 2012).

COMBINED MODELS: IN VITRO DIGESTION CELL-BASED ASSAY

In recent studies, the bioactive effects of dairy proteins have been assessed using the protocol reported by Petrat-Melin et al. (2017). Those authors studied the digestion fate of β -CN and the formation of bioactive peptides VYFPGPPIHN and VYFPGPPIP using a pepsin and pancreatin protocol. Based on the digestion results, Petrat-Melin et al. (2017) synthesized the bioactive peptides in vitro and assessed their ACE inhibitory capacity before and after incubation with a monolayer of Caco-2 cells. The ACE inhibitory capacity in vivo can be mediated by the synergistic effect of a combination of peptides or by the whole protein

digestate; therefore, the combination of in vitro digestion models with intestinal cell culture models may increase the physiological relevance of in vitro studies. To date, only a few studies have used combined models with dairy bioactive compounds. Picariello et al. (2013) applied a combined model to investigate the delivery of bioactive milk peptides across monolayers of intestinal Caco-2 cells. Furthermore, Giromini et al. (2016) used a combined in vitro digestion and cell culture model to study the proliferative effect of caseins and whey proteins at the intestinal level. Basiricò et al. (2015) studied the release of ACE inhibitor peptides during in vitro gastrointestinal digestion of Parmigiano Reggiano cheese and their absorption through an in vitro model of intestinal epithelium. Volstatova et al. (2016) combined the application of the INFOGEST protocol to study the effect of milk digesta from bovine, goats, and sheep on *Lactobacillus* adherence to human Caco-2/HT29-MTX intestinal cells. Pepe et al. (2016) was the first to couple the in vitro digestion of Stracchino cheese to intestinal bioavailability and bioactivity studies. The digestate was fractionated by a semipreparative reverse-phase liquid chromatography and tested both for bioavailability and antioxidant properties in an H₂O₂-stressed Caco-2 cell line. The in vitro digestion, together with the intestinal cell absorption, can provide several pieces of information for in vivo correlations. However, the number of in vitro digestion or cell culture studies is limited, and the application of the combined models to study the physiology of dairy protein digestion and bioactive peptide evaluation is at its beginning. The application of the combined models, however, should always be a balanced compromise between biotechnological complexity and physiological relevance (Guerra et al., 2012). Obviously, other parameters should be carefully considered, including the presence of other food components, such as lipids and sugars, as well as the stability of the peptides, which are able to enter systemic circulation. A strict interaction among the gut epithelium, microbiota, and the secondary target tissues exists due to chemical and biological signals mediated by food compounds or their metabolites. The application of multiorgan cell culture models composed of a series of bioreactors simulating the dynamics of the different tissue environment in a single model was proposed by Ahluwalia et al. (2018). Each bioreactor contains a specific cell type and all bioreactors are interconnected by a continual flux of culture medium. These systems allow testing of translocation across epithelial barriers of a compound and the transport of absorbed nutrients to the blood stream circulation or secondary target organs. Even though these systems are in the early stages of development, further investigations should be focused on the development of

Table 4. Intestinal cell lines used for the study of dairy protein and peptide absorption and bioactivity studies; examples of applications

Cells	Origin/cell type/species	Dairy compound/peptide tested	Study outcome	References
Caco-2	Colon/epithelial enterocyte-like/human	β -CN/peptide (193–209)–immunomodulatory	Transport of intact peptide via transcytosis	Regazzo et al., 2010
		Whey protein/peptide ALPMHIR - ACE inhibitory	Transport of intact peptide	Vermeirssen et al., 2002
		Bovine lactoferrin	$\uparrow$ TEER ¹	Anderson et al., 2017
		Casein protein/peptide VPP - ACE inhibitory	Transport of intact peptide via paracellular diffusion	Satake et al., 2002
		Caseins skim milk/peptide panel	Transport of intact peptides	Picariello et al., 2013
		Casein hydrolysate	Transport of intact peptides: via paracellular route [high-molecular weight peptides (1,600–500 Da)]/via PepT1 (low-molecular weight peptides, <500 Da)	Wang and Li, 2017
HT29-MTX	Colon/epithelial goblet-like/human	Parmigiano Reggiano cheese/peptides VPP, IPP, RYLGY, RYLQ, AYFYPEL, AYFYPE, LHLPLP, and LPLP ACE-I inhibitory	Transport of intact VPP, IPP, AYFYPEL, AYFYPE, LHLPLP, and LPLP	Basiricò et al., 2015
		β -CN/ β -casomorphin-5 and -7 and casoxin-6- μ -opioid agonist and antagonist	Transport of intact peptides	Sienkiewicz-Szapka et al., 2009
		κ - and β -CN/peptides NPYPVR, AVYPYQR, KVLVPVEK, and ARHPHLSFM	Antioxidant effect at intestinal cell level	Tonolo et al., 2018
		β -CN/peptide KVLVPVEK-antioxidant	Transport of intact peptide	Tonolo et al., 2018
		β -LG/peptide RLSFNP	Transport of intact peptide via the paracellular route	Guo et al., 2018
		WPC hydrolysate/ β -lactoraphin	$\uparrow$ MUC5AC ⁴ expression and mucin	Martínez-Maqueda, et al., 2013a
		α _{S1} -CN/AYFYPEL and YFYPEL peptides	$\uparrow$ MUC5AC ⁴ gene and mucin expression	Martínez-Maqueda, et al., 2013b
		β -CN/peptides (94–108) and (117–123)	$\uparrow$ MUC5AC ⁴ , MUC2 ⁶ expression	Plaisancié et al., 2015
		Fermented milk (yogurt)/total peptide pool (TPP)	$\uparrow$ MUC4 ⁵ , MUC2 ⁶ expression	Plaisancié et al., 2013
		β -Casomorphin-7	$\uparrow$ MUC5AC ⁴ gene and mucin expression	Zoghbi et al., 2006
IPEC-J2	Jejunum/epithelial enterocyte-like/pig	α _{S1} -CN/YFYPEL peptide	$\uparrow$ MUC5AC ⁴ expression	Fernández-Tomé et al., 2016
		β -Casofensin	Protects intestinal epithelial cells against apoptosis	Besette et al., 2016
		Whey and casein proteins digestates	Cell proliferation modulation	Giromini et al., 2016
IPEC-I	Jejunum and ileum/epithelial/pig	Colostrum and cheese whey	$\uparrow$ Cell proliferation	Blais et al., 2014
		Casein glycomacropptide	$\uparrow$ /Gene expression involved in cell proliferation, adhesion or migration	González-Ortiz et al., 2013
T84 IEC-6	Colon/epithelial/human Small intestinal/epithelial-like/rat	Lactoferrin/LFP-20 peptide	Attachment of <i>E. coli</i> <i>Escherichia coli</i>	Zong et al., 2016
		whey protein digestate	$\uparrow$ Zo-1 ² , claudin-1 and occludin	
		Casein and whey hydrolysates	$\uparrow$ TEER ¹ (under LPS stress condition) $\uparrow$ Cell proliferation $\uparrow$ Cell migration $\uparrow$ Cell proliferation	Morgan et al., 2014 Purup et al., 2018

Continued

Table 4 (Continued). Intestinal cell lines used for the study of dairy protein and peptide absorption and bioactivity studies; examples of applications

Cells	Origin/cell type/species	Dairy compound/peptide tested	Study outcome	References
STC-1 pGIP/neo	Enterococcine/epithelial-like/mouse	β-LG and α-LA	↑Cell proliferation ↑Incretin hormones glucagon-like peptide-1 secretion	Gillespie et al., 2015
PSIc1	Small intestinal epithelial-like/pig	Whey protein concentrate	↑Cell proliferation and cytokine responses	Nguyen et al., 2016.

¹TEER = trans-epithelial electrical resistance.
²Zo-1 = zonula occludens-1.
³ETEC K88 = enterotoxigenic *Escherichia coli* K88.
⁴MUC5AC = mucin 5AC gene.
⁵MUC4 = mucin 4 gene.
⁶MUC2 = mucin 2 gene.

complex in vitro systems, which can be used before or in parallel to in vivo intervention studies.

CONCLUSIONS

The most important health benefits of dairy proteins are obtained following gastrointestinal digestion and peptide release. Static and dynamic digestion models that apply simulated human digestion conditions are becoming valuable tools for the estimation of preabsorptive events (i.e., digestibility, bioaccessibility) of dairy proteins and peptides. Further improvements could be made to make these systems even more relevant, such as coupling the digestion models with intestinal cell culture to simulate the epithelial transport and the intestinal bioactive properties of the dairy peptides and AA obtained as a digested mixture. Such an approach can overcome the major limitation of using synthetic peptides in cell-based bioassays. According to the principle of replacement, reduction, and refinement, the latter approach may limit the number of experimental animals used during in vivo trials and facilitate the study of the mechanism of action of specific proteins and peptides and the relative digestion metabolites. Dairy peptides primarily modulate the intestinal epithelium (bioaccessible fraction), but after intestinal absorption, they may influence the function and the physiology of other tissues and organs (bioavailable and bioactive fraction). In vitro digestion models in association with a cell-based bioassay provide an enormous boost to the practical and economic potential of dairy protein bioactivity measurements. The increasing sophistication of in vitro models has several advantages from ethical, economical, and scientific perspectives, even though it remains unavoidable to extend the results obtained in vitro to appropriate in vivo bioavailability assays to track the metabolic fate of dietary proteins and to validate the actual involvement of dairy peptides in physiological events.

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