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# Review: Harmonised *in vitro* digestion and the Ussing chamber for investigating the effects of polyphenols on intestinal physiology in monogastrics and ruminants

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ABSTRACT

Because of the relevant effects of plant-derived polyphenols (**PPs**) on monogastrics and ruminants' nutrition, emissions and performance, an increasing number of *in vivo* and *in vitro* studies are being performed to better understand the mechanisms of action of polyphenols at both the ruminal and intestinal levels. The biological properties of these phenolic compounds strongly depend on their degradation, absorption and metabolism. The harmonised *in vitro* digestion method (**INFOGEST**) is one of the most reliable *in vitro* methods used to assess the bioaccessibility and or antioxidant activity of PP contained in different matrixes, as well as the interactions of PP and their degradation products with other feed ingredients. The effects of PP released from their matrix after *in vitro* digestion on different intestinal physiological parameters, such as epithelium integrity, can be further evaluated by the use of *ex vivo* models such as the Ussing chamber. This review aims to describe the combination of the INFOGEST method, coupled with the Ussing chamber as a valuable model for the digestion and subsequent effects and absorption of phenolic compounds in monogastrics and potentially in ruminants. The advances, challenges and limits of this approach are also discussed.



## Implications

*In vitro* digestion protocols are widely used to address questions in the field of nutritional research, such as the interactions between nutrients and bioactive compounds. Different absorption models can be used to further investigate the fate of polyphenols and their metabolites released by the food matrix after digestion. The information provided by the present review will help scientists to make the correct decision about the proper model for the digestion, absorption, bioaccessibility and biological effects of phenolic compounds from different matrixes.

## Introduction

The bioaccessibility and bioactivity of plant polyphenols (**PPs**) in the small intestine vary depending on the intestinal pH, temperature, bile salts concentration, digestive enzymes activity and the changes in the chemical structures due to the stomach environment, which then may affect their interactions with dietary nutrients (Nagar et al., 2021) or the gut microbiome or both (Tretola et al., 2019).

To better understand the interactions of PP and their degradation products with dietary nutrients, a combination of a harmonised *in vitro* digestion method (**INFOGEST**) system (Brodkorb et al., 2019) and an INFOGEST method with Ussing perfusion chambers might be a way to shed light on these complex interactions. Because of the dynamic processes, which involve complex enzymatic and physiological events that take place in different gastrointestinal segments, the use of a single model to reproduce the digestion and absorption of nutrients *in vitro* is difficult to simulate.

*In vitro* digestion (**IVD**) protocols are widely used to address questions in the field of nutritional research (such as interactions between nutrients and bioactive compounds), as they are cheaper, faster, simpler to perform than *in vivo* experiments and do not imply ethical questions. The IVD protocols have better repro-

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ducibility and fewer inter-individual variabilities and are therefore optimal for screening experiments. However, the *in vitro* simulation of complex intestinal absorption is challenging. In the digestive tract of monogastrics, around 90% of the absorption occurs in the small intestine and the protein expression of the transporters involved in nutrient absorption varies along the small intestinal tract, increasing the complexity of the analysis (Balimane and Chong, 2005). Thus, the biological relevance of the IVD data needs to be validated *in vivo* for each research question.

Because of the relevant effects of PP on ruminant nutrition, emissions and performance, an increasing number of *in vivo* and *in vitro* studies are being performed to test the effects of PP like tannin-rich feed or pure tannin extracts (such as tannins from forage legumes) or other phenolic compounds (e.g., quercetin, a flavonoid) at both the ruminal and intestinal levels (Min et al., 2020).

Considering the intestinal environment and its dynamic conditions, the ideal intestinal *in vitro* model should contain several features, the most important being the cell culture type, which needs to be representative of the native gut epithelium. Further aspects to be addressed are adequate oxygenation and nutrient level; an environment that comprises the epithelium-immune system crosstalk; and finally, the host-gut microbiota interaction (Brodkorb et al., 2019).

Several *in vitro* methods are used to mimic intestinal absorption. They can involve immortalised cell lines, primary cells, intestinal organoids (or Mini-Guts), 3D architecture, multilayer models, bioreactors, millifluidic chambers and microfluidic devices. All these approaches are extensively described in the review of Costa and Ahluwalia (2019).

The present review aims to describe the combination of INFOGEST, coupled with Ussing chamber, as a valuable model for the digestion and subsequent absorption of nutrients or bioactives in monogastrics and ruminants, with special emphasis on phenolic compounds. The provided information will help the reader judge the level of complexity needed to answer specific effects on digestion and/or absorption processes for evaluating the effects of bioaccessible polyphenols on intestinal epithelial integrity. The developments, challenges and limits of this approach will be discussed.

## Digestion and absorption of phenolic compounds

Plant food bioactives are widely used to reduce the risks of chronic diseases in humans (Manach et al., 2017) and to reduce the use of antibiotics in animals (Girard and Bee, 2020), in addition to many other applications (Tretola et al., 2019). PP compounds, such as tannins, are probably the most investigated compounds because of their applications to human and animal health. Tannins are classified into three categories: condensed tannins (**CTs**), hydrolysable tannins (**HTs**) and complex tannins. The properties of these phenolic molecules strongly rely on their absorption and metabolism, which depend in turn on the food matrix and the digestion process (Barry et al., 2021). Other aspects such as the gut microbiota composition and the individual metabolic status can impact the PP properties (Aravind et al., 2021).

### *Monogastrics*

The PP cannot be absorbed in their native forms, but they require hydrolysis by digestive enzymes, intestinal microbial population or both (Girard and Bee, 2020). Because of their lipophilic nature, only the aglycones (such as flavan-3-ols and gallic acid) can interact with the membranes of the enterocytes. Several studies have reported the effects of the food matrix on gastrointestinal polyphenol release as well as the efficacy by which they are transported across the mucosal epithelium (Tagliazucchi et al., 2010; Mandalari et al., 2016). It is known that polyphenols interact with other molecules (mainly protein and fat). These complexes affect the bioaccessibility of phenolic acids (Mandalari et al., 2013).

As previously mentioned, pH, temperature, bile salt concentration and digestive enzyme activity can affect the bioaccessibility of PPs (Di Lorenzo et al., 2021). Therefore, different IVD models have been developed to simulate intestinal physiology in a more controlled environment, compared to *in vivo* models (Minekus et al., 2014; Brodkorb et al., 2019; Mulet-Cabero et al., 2020a). The first step of PP digestion takes place in the oral phase. Studies showed that salivary α-amylase is not involved in the release of phenolic compounds, since the simulated oral digestion with α-amylase did not affect the amount of phenolic compound released compared to the undigested counterpart (Minekus et al., 2014). However, glycoside flavonoids are subjected to the hydrolyses mediated by the β-glycosidase. The efficacy of this step depends on the type of sugar present in the phenolic molecule, with the glucose conjugates being the most hydrolysed (Teng and Chen, 2019; Girard and Bee, 2020).

Oligomers released in the mouth are degraded to smaller units in the stomach due to the acidic pH of the gastric juices. Aglycones such as the flavon-3-ols then pass intact into the duodenum where (like in the other segments of the small intestine) reactions such as deglycosylation, glucuronidation, methylation and hydroxylation of flavonoids occur (Teng and Chen, 2019). Because of the neutral pH, oxidation of the flavonoid epigallocatechin gallate may occur, leading to a potent form for scavenging free radicals (Teng and Chen, 2019).

Absorption of free phenolic acids is another event that can take place in the small intestine (even if the majority of phenolic compounds reach the large intestine), mainly in the form of esters of phenolic acids (Girard and Bee, 2020). Because of their poor intestinal absorption, large quantities of polyphenol compounds are delivered to the colon, where many undergo extensive metabolism via the colonic microbiota (Mosele et al., 2015). In particular, colonic bacteria hydrolyse glycosides to aglycones, and subsequently transform them into various acids through the action of bacterial β-glucosidase, β-rhamnosidase and esterases, which degrade the flavonoid chains into singular units.

Therefore, a heterogeneous variety of metabolites can be produced, depending on the structure of polyphenols. Once absorbed, polyphenols are extensively metabolised in tissues, where they are conjugated to form O-glucuronides, sulphate esters and O-methyl ether (Scott et al., 2022). A large proportion of these are subsequently secreted with bile back into the gut, where they can be subjected to the action of the colonic microbiota and absorbed again or excreted via the faeces.

In humans, data on absorption, distribution, metabolism and bioavailability showed that the maximum concentration of anthocyanin and its glycoside in plasma ranged from 11.4 to 110.8 μg after the intake of 500 mg of cyanidin-3-glucoside (one of the main anthocyanins in red raspberry) for 0.5–24 h (Czank et al., 2013). Because of low intestinal absorption, some researchers have questioned the ability of ingested dietary polyphenols to affect systemic antioxidant capacity (Sesso et al., 2003; Halliwell et al., 2005). However, studies have suggested that intestinal luminal concentrations of polyphenols might be much higher than serum concentration (Dryden et al., 2006), exerting their effects on the gut microbiota (Tretola et al., 2019) but also intestinal epithelial integrity and nutrient uptake (Tretola et al., 2020).

In the light of this evidence, combining *in vitro* digestion with an absorption model to reproduce the intestinal epithelium could be a way to shed light on the complex interaction between PP and their degradation products, not only on specific digested

nutrients such as amino acids, peptides, tri- and di-monoglycerides and fatty acids but also on absorption processes.

*Ruminants*

The occurrence of PP in herbivores' diets is very common, especially for grazing animals. For instance, Fraisse et al. (2007) estimated that a grazing cow could consume up to 500 g per day of PP. Recent evidence has promoted the use of tannins in ruminant diets because of their positive impact on rumen microbial activity, ruminal fermentation rate, antioxidant status and health of ruminants (Orzuna-Orzuna et al., 2021).

Tannins also shift N excretion from urine to faeces by modulating nitrogen metabolism in the rumen, abomasum and intestine (Brinkhaus et al., 2016).

The efficiency of ruminant livestock mainly depends on the quantity of protein delivered from the rumen, where the protein reaching the abomasum represents the sum of dietary and microbial proteins. To ensure a high amount of N availability for intestinal absorption, low proteolysis and high efficiency of microbial protein synthesis are required in the rumen. Lower dietary protein degradation in the rumen results in a reduction in ruminal ammonia production (which is in part excreted by the urine) and in higher protein digestion in the small intestine.

Due to their ability to bind proteins, tannins have been found to modulate rumen fermentation. Although the literature is not consistent, most studies found that tannins reduce protein degradation in the rumen, prevent bloating and inhibit methanogenesis (Patra and Saxena, 2011), with the latter aspect still being the subject of controversial studies (Lazzari et al., 2021). The reduction of protein degradation in the rumen is probably due to the formation of tannin–protein complexes (at rumen pH) from both CTs and HTs.

The direct effect on microbial proteolytic enzymes that can be inhibited by tannins is another mechanism that could lead to the reduction of protein degradability in the rumens of animals fed tannins (Patra and Saxena, 2011). These actions depend on the properties of the tannins, with gallic acid exerting no or low effects on protein degradability and tannic acid having a strong protein protective effect in the rumen. Moreover, only polymers are bioactive in this regard. It has been found that CTs-protein complexes cannot be dissociated by ruminal bacteria, which is not true for HTs-protein complexes. For both CT- and HT-protein complexes, the dissociation always takes place at pH < 3.5 (e.g., in the abomasum) and pH > 7 (e.g. in the small intestine), where the proteins become available again for intestinal digestion and absorption (Al Kindi, 2015). However, not all the complexes were dissociated at postruminal pH. In this case, a higher amount of faecal N excretion can be observed.

Studies have demonstrated that different classes of tannins can affect intestinal amino acid uptake (Nawab et al., 2020). The increased faecal N extraction could also be due to the ability of un-degraded free tannins to bind again to proteins at the large intestine pH (5.5–7.0), thus decreasing the recycling of the N and increasing its excretion through the faeces.

The N in faeces and urine is a source of nitrogenous compounds, a substrate used by microorganisms in the soil to produce $N_2O$ by nitrification and denitrification processes (Zhou et al., 2019). The latter represent a potent source of greenhouse gases (**GHG**), with a strong warming potential. Previous studies have indicated that the ability of tannins to create stable complexes with proteins can be used to protect feed CP from rumen microbial degradation and increase the amount of rumen undegradable CP (Zhou et al., 2019). At high dietary CP levels, urinary N extraction increases in cattle, so the ability of tannins to reduce dietary CP microbial degradation can lead to reduced urinary N extraction, thus decreasing $N_2O$ emissions from urine (Zhou et al., 2019).

Urinary N extraction has a bigger impact on ammonia production from ruminants compared to the less volatile form of the CT-protein complex in faeces (Zhou et al., 2019). Tannins have no or negligible effects on soluble carbohydrate digestibility. Contrasting results have been obtained *in vitro* compared to *in vivo* studies. Different tannins have been found to affect DM digestibility to a different extent, depending on the category of the tannin (Patra and Saxena, 2011).

## The harmonised *in vitro* digestion model

*In vitro* digestion methods can generally be divided into static and dynamic models. Dynamic models are more complex, laborious and expensive compared to static models. Static *in vitro* digestion models are highly predictable in the outcome compared to *in vivo* trials, as the environmental factors can be standardised and genetic variability does not play a role.

*In vitro* digestion models have been used to study several aspects, such as the matrix release of micronutrients or secondary plant compounds including polyphenols (Brodkorb et al., 2019). *In vitro* digestion methods such as INFOGEST are used to produce bioaccessible fractions that can then be used to address questions (such as intestinal integrity or transport by employing different absorption models), as described below.

The various IVD protocols in use differ in several key variables—such as the chemical composition of the simulated digestive juices; incubation time and temperature; and type, amount and activity of digestive enzymes—leading to a very difficult comparison of results between different laboratories. The "harmonised static *in vitro* simulation of the gastrointestinal food digestion" is a monogastric *IVD* protocol established by a network of multidisciplinary experts from more than 35 countries (Brodkorb et al., 2019).

The procedure of the INFOGEST protocol comprises preparation, digestion and sample treatment or analysis phases. Brodkorb et al. (2019) describe each phase in detail. Briefly, in the INFOGEST protocol, the digestive phases of the dynamic *in vitro* method are divided into oral, gastric and intestinal digestion, each characterised by specific experimental conditions and involving specific enzymes.

The oral phase includes simulation of the salivary fluids with or without salivary amylases, depending on the matrix subjected to digestion. The duration of this phase is 2 minutes at pH 7, after which the oral bolus is diluted with simulated gastric fluids and subjected to digestion by gastric enzymes (pepsin and gastric lipase) for 2 h at pH 3.0. The gastric chime is then diluted again with simulated intestinal fluids, bile salts and pancreatic enzymes (pancreatin based on the activity of trypsin or as individual enzymes) and incubated at pH 7 for an additional 2 h. The experimental conditions (pH, time of digestion, enzyme activity, etc.) were chosen by the authors according to physiological data described in detail in (Minekus et al., 2014). In the last step of digestion, samples are collected, treated, stored and subsequently analysed.

Compared to other IVD protocols, one key parameter of the INFOGEST method is the standardisation of the activity of the digestive enzyme cocktail and the concentrations of bile salts (Minekus et al., 2014). By doing so, the reproducibility and repeatability of this method can be greatly improved, as both the enzyme activity and the bile salts are fundamental factors.

*Harmonised in vitro digestion method and polyphenols*

The bioaccessability and/or antioxidant activity of PP contained in different matrices were tested using the INFOGEST system. Recent studies focused on different matrices are reported in

**Table 1**
INFOGEST *in vitro* digestion studies aiming to investigate the effects of *in vitro* digestion on polyphenol bioaccessibility or the effects of digested phenolic compounds on digestive processes of other nutrients in monogastrics and ruminants.

| Polyphenol sources | Aim | Main outcome | Year | Reference |
|---|---|---|---|---|
| Apple (*Malus domestica*) | Evaluation of the impact of the *in vitro* digestion on polyphenols bioaccessibility and antioxidant properties of different varieties of apple (*Malus domestica*). | *In vitro* digestion favours the release of phenolic compounds. Their content was lower than that obtained before *in vitro* digestion. Chemical extraction could overestimate the bioavailability of phenolic compounds. | 2021 | Corona-Leo et al., 2021 |
| Apples (Annurca, Limoncella, Red Delicious, and Golden Delicious) | Assessment of *In vitro* bioaccessibility of polyphenols from Annurca, Limoncella, Red Delicious, and Golden Delicious apples using a sequential enzymatic digestion model. | The reproduction of colonic biochemical conditions breaks the dietary fibre-polyphenols interactions and increases the release of polyphenols. | 2021 | Graziani et al., 2021 |
| Beans (*Phaseolus vulgaris*) | Starch digestion effects on bioaccessibility of polyphenols from beans (*Phaseolus vulgaris*). | The bioaccessibility of bean polyphenols is strongly enhanced by starch digestion. | 2020 | Perez-Hernandez et al., 2020 |
| Berry | Interaction of bread and berry polyphenols on starch digestibility and Polyphenols' bioaccessibility. | PP interaction with starch reduces PP bioaccessibility, reducing the amount of PPs available for α-amylase inhibition. The PPs–starch interaction inhibits starch digestion. | 2020 | Kan et al., 2020 |
| Buckwheat flour and amaranth flour | *In vivo* and *in vitro* model studies on noodles prepared with antioxidant-rich pseudocereals. | Total PP content and antioxidant capacity continuously increased in the whole gastrointestinal tracts. | 2019 | Kiss et al., 2019 |
| Cauliflower waste | Bioavailability and intestinal mucus diffusion of polyphenols from cauliflower waste. | The recovery of PPs in the gastric phase was approximately 70% lower after the intestinal phase. | 2015 | Gonzales et al., 2015 |
| Chickpea and *Tribulus terrestris* | Inhibitory effects of chickpea and *T. terrestris* on lipase, α-amylase and α-glucosidase. | *T. terrestris* and chickpea are potent inhibitors of key enzymes in digestion of carbohydrates and lipids *in vitro*. | 2016 | Ercan and El, 2016 |
| Coffee grounds | *In vitro* evaluation of the bioaccessibility and antioxidant properties of polyphenols from spent coffee grounds-enriched cookies. | The highest bioaccessibility of spent coffee ground PPs was observed after the colonic stage. | 2021 | Castaldo et al., 2021b |
| Coffee grounds | Antioxidant and anti-inflammatory activity of coffee brew evaluated after simulated gastrointestinal digestion. | Digested coffee reduced interleukin-6 levels compared to the not-digested counterpart. The digestion led to the release of highly bioactive compounds. | 2021 | Castaldo et al., 2021c |
| Coffee pulp | Intestinal bioaccessibility of phenolic and caffeine after INFOGEST digestion model. | The *in vitro* digestion decreased the phenolic compounds with the exception of the caffeine. The bioaccessibility of polyphenols and caffeine was high but not one of the flavonoids. | 2022 | Cañas et al., 2022 |
| Fennel (*Foeniculum vulgare* Mill.) | Chemical composition, *in vitro* bioaccessibility and antioxidant activity of polyphenolic compounds from nutraceutical fennel waste extract. | Acidic gastric conditions negatively affected the polyphenol compounds released. | 2021 | Castaldo et al., 2021a |
| Galician extra-virgin olive oil | Investigate the bioaccessibility of polyphenols from Galician extra-virgin olive oil. | Gastric digestion generated free tyrosol, hydroxytyrosol and hydroxytyrosol acetate from secoiridoids after intestinal digestion, simple phenols were released and mainly recovered in the water phase. | 2021 | Reboredo-Rodriguez et al., 2021 |
| Grape extracts | Effect of the food matrix on polyphenol bioaccessibility and antioxidant activity. | Food matrices protect anthocyanins from degradation during the intestinal phase but had no effect on antioxidant capacity. | 2016 | Pineda-Vadillo et al., 2016 |
| Honey from Sicilian black honeybee (*Apis mellifera* ssp. sicula) | Antiproliferative effects of bioaccessible fractions of honeys from Sicilian black honeybee on human colorectal carcinoma cells. | Despite the considerable decrease in total polyphenols occurred after digestion, the combination of phytochemicals present in the bioaccessible fractions still provides anticancer effects indicating the importance to check a whole matrix subjected to a simulated gastrointestinal digestion. | 2022 | Cilla et al. 2022 |
| Lettuce (*Lactuca sativa* L.) | Bioaccessibility of polyphenols and antioxidant capacity of fresh or minimally processed modern or traditional lettuce (*Lactuca sativa* L.) varieties. | Accumulation of phenolic compounds after minimal processing was matrix-dependent. The quantity of bioaccessible polyphenols was higher after minimal processing and storage. | 2020 | Lafarga et al., 2020 |
| Lipophilic polyphenol compounds | Encapsulation of lipophilic polyphenols in plant-based nanoemulsions: impact of carrier oil on lipid digestion and curcumin, resveratrol and quercetin bioaccessibility. | Some of the lipophilic polyphenol compounds inhibited lipid digestion for certain oil types. Resveratrol retarded the digestion of coconut, sunflower and flaxseed oils, but it still had the highest gastrointestinal stability and bioaccessibility. | 2021 | Zhou et al., 2021 |
| Olive pomace | Influence of pomace matrix and cyclodextrin encapsulation on olive pomace polyphenols' bioaccessibility and intestinal permeability. | High bioaccessibility but relatively low permeability of olive pomace extracts' polyphenols, which was negatively affected by the matrix. | 2020 | Radić et al., 2020 |
| Olive pomace | Simulated digestion of an olive pomace water-soluble ingredient: relationship between the bioaccessibility of compounds and their potential health benefits. | The *in vitro* gastro intestinal digestion of the liquid-enriched olive pomace powder decreases the bioaccessibility and antioxidant activity of the polyphenols. | 2020 | Ribeiro et al., 2020 |

**Table 1** (*continued*)

| Polyphenol sources | Aim | Main outcome | Year | Reference |
|---|---|---|---|---|
| Olive pomace extract | | The bioaccessibility and transepithelial permeability of olive pomace extract-derived polyphenols can be significantly affected by foods (nutrients), especially by casein and some dietary fibre. These effects are polyphenol-and nutrient-specific and are achieved either through complexation in the gastrointestinal lumen and/or through direct effects of nutrients on the intestinal monolayer. | 2020 | Vitali Čepo et al., 2020 |
| Plant sterol-enriched milk-based fruit beverages | Impact of galactooligosaccharides on the bioaccessibility of sterols in a plant sterol-enriched beverage. | The addition of galactooligosaccharides did not affect total plant sterol bioaccessibility. | 2018 | Blanco-Morales et al., 2018 |
| Raspberry (*Rubus idaeus*), boysenberry (*R. ursinus* × *R. idaeus*), redcurrants (*Ribes rubrum* sp.) and blackcurrants (*Ribes nigrum* sp.). | Berry fruits-enriched pasta: effect of processing and *in vitro* digestion on phenolics and its antioxidant activity, bioaccessibility and potential bioavailability. | Raspberry and boysenberry can reduce the glycaemic response to pasta products through *in vitro* digestion, retarding starch hydrolysis (probably due to inhibition of digestive enzymes), promoting an increase in slowly digested starch and reducing the total starch hydrolysed. | 2020 | Bustos et al., 2020 |
| Ripe berries from the species *Gaultheria phillyreifolia, G. poeppigii* pink fruits and *G. poeppigii* white fruits | Iridoids and polyphenols from Chilean *Gaultheria* spp. berries effects on the glucose uptake in Caco-2 cells after simulated gastrointestinal digestion. | The simulated digestion decreases the total content of anthocyanins by 98–100%, flavonols by 44–56%, phenylpropanoids by 49–75% and iridoids by 33–45%. Digested extracts inhibited α-glucosidase and decreased glucose uptake in Caco-2 cells. Moreover, decreased mRNA expression of glucose transporters SGLT1, GLUT2, GLUT5. | 2022 | Mieres-Castro et al., 2022 |
| Tea polyphenol extract | Binding of tea polyphenols to soy proteins and effects on pepsin diffusivity and *in vitro* gastric digestion of soymilk. | The binding between soy proteins and tea polyphenols significantly impaired *in vitro* gastric digestion of soymilk by decreasing pepsin diffusivity. | 2021 | Ge et al., 2021 |
| Terebinth (*Pistacia terebinthus* L.) coffee | Influence of milk, sugar and sweetener addition on bioaccessibility of terebinth (*Pistacia terebinthus* L.) coffee polyphenols. | The addition of whole milk to terebinth coffee increased the total bioaccessible flavonoids significantly (45%) after the *in vitro* digestion, whereas skim milk addition did not result in any significant change. | 2022 | Kamiloglu et al., 2022 |
| White, green and black tea | Colon bioaccessibility and antioxidant activity of white, green and black tea polyphenols' extract after *in vitro* simulated gastrointestinal digestion. | After simulated gastrointestinal digestion, the bioaccessibility in the colon stage was significantly increased compared to the duodenal stage for both tea polyphenols and total phenol content. Similarly, the antioxidant activity in the colon stage was significantly higher than that in the duodenal stage. | 2018 | Annunziata et al., 2018 |
| Whole apple | Effects of whole apples on lipid digestibility and bioaccessibility of a high dairy fat meal. | The presence of apples did not alter milk fat lipolysis in the static *in vitro* digestion model but reduced milk fat bioaccessibility dynamic conditions. | 2021 | Lin et al., 2021 |
| Wild and commercial blackberries (*Rubus* spp.) | Impact of *In vitro* gastrointestinal digestion on stability, bioaccessibility and antioxidant activity of polyphenols from wild and commercial blackberries (*Rubus* spp.). | After *in vitro* gastrointestinal digestion, the total phenolic and anthocyanin contents in blackberries decreased by ≥68% and ≥74%, respectively. More than 40 phenolics were identified during digestion; most of them degraded completely during digestion. Gastrointestinal digestion had a negative effect on the antioxidant activity of both fruits. | 2021 | Sánchez-Velázquez et al., 2021 |

Note: The table indicates the sources of polyphenols, the aim, the main outcome, the year of publication and the reference of each study article.
Abbreviations: INFOGEST = harmonised *in vitro* digestion method; PP = plant polyphenol; SGLT1 = sodium-glucose cotransporter 1; GLUT2 = glucose transporter 2; GLUT5 = glucose transporter 5.

Table 1. The INFOGEST system also allows us to assess the interactions of PPs and their degradation products with other feed ingredients. For instance, Kamiloglu et al. (2022) investigated the impact of the matrix on the bioaccessibility of coffee polyphenols. Coffee formulations were prepared with whole or skimmed milk, with or without sugar/sweetener to study the matrix effect on the bioaccessibility of coffee polyphenols (Kamiloglu et al., 2022). The results revealed that the addition of whole milk to terebinth coffee increased the total bioaccessible flavonoids, whereas skim milk addition did not result in any significant change (Kamiloglu et al., 2022).

If the food/feed matrix can influence the polyphenols' bioaccessibility, then polyphenols can also affect the digestibility of other food/feed matrixes, as shown by Kan et al. (2020), who investigated the effects of berry polyphenols on starch digestion *in vitro* both by co-digestion of berry extract with bread or by fortifying bread with berry extracts. Their results showed that the co-digestion of bread with berry extracts significantly reduced the rate and extent of starch digestion. In addition, the interactions of polyphenols with the matrix reduced the polyphenols' bioaccessibility, thus reducing the quantity of polyphenols available for α-amylase inhibition.

## *In vitro* intestinal absorption models

The gastrointestinal tract (**GIT**) represents the largest interface between the body and the environment. The most abundant cell

types composing the intestinal epithelial layer are enterocytes that exert a strong absorptive function. Other cell types present at the intestinal level are the mucin-secreting goblet cells and peptide hormone-exporting enteroendocrine cells, as well as Paneth cells secreting digestive enzymes, growth factors and antimicrobial peptides. A large number of cells associated with the immune function are present at the intestinal level, such as Peyer's patches, isolated lymphoid follicles, mesenteric lymph nodes, dendritic cells, T-cells, M-cells and lymphoid cells (Faria et al., 2013). This complexity makes it difficult to mimic the nutrient uptake by *in vitro* models (Mackie et al., 2012).

Different *in silico*, *in vitro* or *ex vivo* models can be used to understand the food-intestine interaction and the bioaccessibility of bioactives. Such methods are in deep described in (Thuenemann, 2015).

The monolayer or co-culture of human or animal intestinal epithelial cells (e.g. Caco-2 and HT-29) can be considered the most simple *in vitro* model. Such models can be very useful for investigating different aspects related to intestinal physiology, such as transport mechanisms (e.g. activation of intestinal channels) or cell viability (Béduneau et al., 2014). However, because of their lack of complexity as the one that characterise the GIT, the use of intestinal tissue are preferable for studying the effects of food and food compounds.

Different *in vitro* intestinal tissue models exist for different purposes, as follows. Intestinal rings use intestinal segments cut into small rings to measure the nutrient uptake into the enterocytes. The intestinal segments are constantly in contact with an oxygenated buffer and the tested molecule, with the viability limited up to 60 minutes (Leppert and Fix, 1994). An example of its application is to test the effects of extracts and flavonoids from onion on the glucose uptake employing mice jejunal rings (Schulze et al., 2015).

The everted sac model is a technique that can be used to estimate the ability of food compounds to be absorbed by quantifying the amount of those compounds that can be quantified on the basolateral side and in the cytosol of the epithelial cells. In this method, the buffer and the tested molecules are within the intestinal section with the tissue viability limited up to 2 h (Wilson and Wiseman, 1954). This method has been applied, for example, to test the properties of green tea polyphenols to inhibit the glucose uptake in a mouse model (Kobayashi et al., 2000).

The isolated and perfused intestinal segment model consists in an intestinal segment that includes also the isolated vascular system. Its difficulty and the limited duration of the experiments are limitations of this model (Schwörer et al., 1991). In this review, we focus on the UC as a reliable model to investigate the effects of polyphenols released from their food matrix after INFOGEST *in vitro* digestion on intestinal epithelium integrity. An example of the application of such model is to investigate the disposition of Naringenin, a flavanone found in citrus fruits, in a rat intestinal perfusion model (Xu et al., 2009).

*The Ussing chamber system*

The Ussing chamber device was developed by Danish zoologist Hans Ussing in the 1950s to quantify the ion transport across the skin of frogs (KOEFOED-JOHNSEN and USSING, 1958). The Ussing chambers are increasingly used for the determination of intestinal integrity and nutrient uptake across intestinal tissues or cell culture, but fewer studies have applied the Ussing chamber system to study the impact of PPs on health.

A commercially available 'classic' Ussing chamber system comprises an electronic device, acrylic perfusion chambers, voltage and current electrodes, and software (Fig. 1). The electronic device is responsible for measuring several parameters, such as the potential difference (**PD**) across the epithelium, the short-circuit current (**Isc**) and the transepithelial resistance (**TEER**). The TEER is used as a measure of the thickness of the (stripped) mucosal epithelium as the amount of tissue will influence the amount of ion transport. The Isc is a powerful method to measure the transepithelial ion transport across epithelial membranes and depends on the activation of ion channels. The PP can activate specific ion channels, such as TRP1a for mucosal secretions (Fothergill et al., 2016). Bacterial toxins potentially present at the intestinal level could also exert the same effect and could be considered a confounding factor during data interpretation (Viana, 2016). A sufficiently long period of adaptation of the tissues in the Ussing chamber and the record of the baseline values before the treatment with PP is essential to minimise this confounding factor".

The chambers are made of solid acrylic material divided into two halves, with ports in each half, to connect the circulation system and the electrodes. A slide in which cells or tissues can be mounted is allocated in the middle of the perfusion chamber. One face of a single half of the slide has sharp pins on its surface to facilitate tissue mounting. These pins allow for the puncturing and positioning of an epithelium membrane within the chamber. Each chamber half also has a separate air/gas inlet to drive the circulation system. Gas, commonly a 95% $O_2$/5% $CO_2$ mixture, is forced at low pressure through the inlets into the buffer solutions contained in the two sides of the chamber. The circulating bubbles oxygenate the buffer and improve the mixing of agents added at the apical and/or basolateral sides during the experiment. The support for the chambers includes access to the water jacket so that temperatures can be chosen and set as desired, allowing the temperature of the perfusion solutions to remain constant.

For each chamber, two AgCl and two Ag electrodes are used to measure the voltage and the current, respectively. Those electrodes need to be placed in tips filled with agarose salt bridge and connected to the Ussing chamber. Finally, the software remotely controls the electrodes and acquires and analyses the data. A schematic representation of all parts of the Ussing chamber model system (Physiologic Instruments, San Diego, CA, USA) is shown in Fig. 1. Intestinal segments from different species have been used to perform Ussing chamber experiments. For studies regarding drug metabolism, intestinal biopsies from healthy humans are commonly used (Li et al., 2021). For studies involving animal nutrition or animal efficiency, intestinal tissue from pigs is the most used (Baker, 2008). Compared to monogastrics, fewer studies have been performed on small or large ruminants. In ruminants, the rumen, omasum and abomasum can be mounted on Ussing chamber as well, depending on the research aim.

For intestinal segments of both monogastric and polygastrics, perfusion chambers with an exposure area of 1 $cm^2$ are commonly used. For Ussing chamber studies with rumen tissues originating from large ruminants, the exposure area is much larger (3 $cm^2$), while for small ruminants like sheep and/or goats, a smaller exposure area of 0.95 $cm^2$ seems sufficient, as reported in the literature (Lang and Martens, 1999). The reason for this variability in the exposure area is related to the dimension of the rumen villi, where a 1 $cm^2$ exposed area may not be enough to properly represent all the variability present along the rumen villi.

Independently of the species, the tissue viability needs to be tested at the end of each experiment. It can be tested by adding to the serosal chamber a certain concentration of the cAMP-dependent $Cl^-$ secretagogue forskolin. A forskolin response can be identified by an increase in Isc or in a drop in the potential difference. Only tissues responding to the forskolin must be considered for data analysis. Other molecules such as the carbachol are also used to test the tissue viability. However, tissues obtained from different animal species could respond differently to these molecules. Therefore, to test the tissue viability, the responsiveness

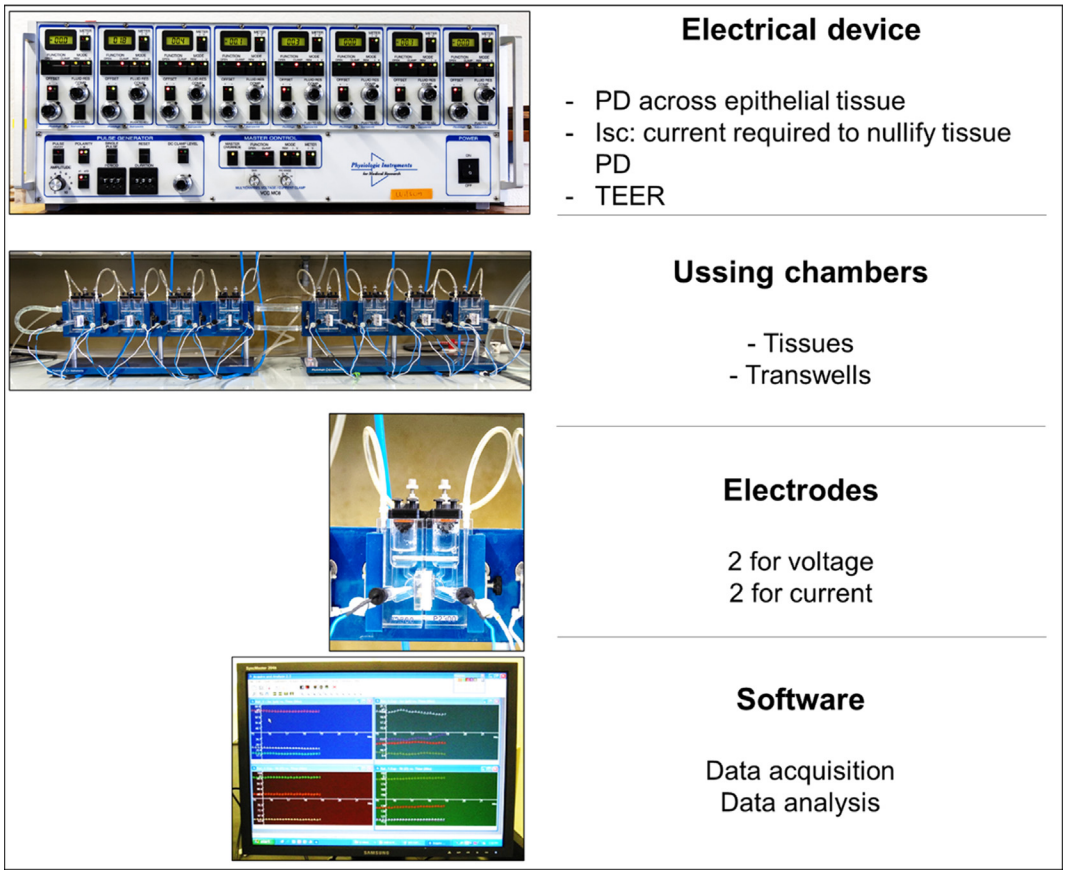


**Fig. 1.** Schematic representation of the Ussing chamber components (Physiologic Instruments, San Diego, CA, USA) adapted to tissues from monogastrics and ruminants. PD: potential difference; Isc: short-circuit current; TEER: transepithelial resistance. Photo credits: Johann Marmy (Agroscope).

of the target tissue to the aforementioned molecules needs to be evaluated before performing the experiments.

When animal tissues are not available or the study design does not necessarily require the complexity of *ex vivo* tissues, different cell cultures seeded on transwells can be mounted in the Ussing chamber. For instance, we investigated the effects of gallic acid on tight junctions and intestinal nutrient uptake through intestinal porcine enterocyte cell line-J2 (IPEC-J2) cells and porcine middle-jejunum segments (Tretola et al., 2020).

Despite this study not being designed to specifically evaluate differences between IPEC-J2 and jejunum tissues, some similarities in the evaluation of gallic acid effects on nutrient uptake were observed (Tretola et al., 2020). However, the *in vitro* model seemed to be more sensitive to gallic acid compared to *ex vivo* models using native intestinal segments, but one must take into account the fact that different experimental conditions were applied.

Examples of studies that used the Ussing chamber model to investigate the effects of polyphenols on different cell lines or tissues obtained from different animal species are reported in Table 2. Note that no studies involving the Ussing chamber and focused on the effects of polyphenols on rumen and on the intestine of large ruminants were found in the literature.

**Table 2**
List of studies published in peer-reviewed journals in which the Ussing chamber model has been employed on cell cultures or a specific gastrointestinal segment obtained from pig, rat, sheep, chicken or fish. All the studies reported in the table refer to the effects of polyphenols on intestinal integrity and physiology.

| Species | Cell line/Tissue | References |
|---|---|---|
| Cell lines | T84 | Schuier et al., 2005; Bergmann et al., 2009; Rogoll et al., 2010; Deusser et al., 2020 |
| | Caco-2 | Steinert et al., 2008; Scherbl et al., 2014; Biolley et al., 2019 |
| | HT-29/B6 | Lobo de Sá et al., 2019 |
| | Ipec-J2 | Tretola et al., 2020 |
| Pig | Ileum | Deußer et al., 2013; Hoppe et al., 2018 |
| | Jejunum | Bruins et al., 2006; Erk et al., 2014; Tretola et al., 2020 |
| | SI | Guschlbauer et al., 2013; Klinger, 2020 |
| Rat | Jejunum | Wolffram et al., 2002; Snoussi et al., 2014; Rtibi et al., 2017 |
| | Ileum | Amasheh et al., 2012; Schloesser et al., 2017 |
| | Colon | Amasheh et al., 2012; González-Quilen et al., 2019 |
| | SI | Matsumoto et al., 2005; González-Quilen et al., 2019 |
| Sheep | Rumen | Patra et al., 2019 |
| | Jejunum | Patra et al., 2019 |
| Chicken | Duodenum | Placha et al., 2015 |
| Fish | Posterior intestine | Trischitta and Faggio, 2008 |

Abbreviation: SI, small intestine (both jejunum and ileum).

## Applications of coupled *in vitro* digestion and Ussing chamber models

To date, only a few studies have combined the *in vitro* digestion protocol with the Ussing chamber system to evaluate the absorption of metabolites or their effects on epithelial integrity. These studies usually refer to monogastrics. In a pilot study, Ozorio et al. (2020) digested *in vitro* the whey hydrolysate and tested the intestinal absorption of the peptides released after the simulated digestion by using jejunal segments of piglets mounted in a Ussing chamber. This study disproved the belief that only free amino acids (along with di- and tri-peptides) could be transported across the intestinal epithelium (Miner-Williams et al., 2014). Ozorio and colleagues' study (2020) improved the knowledge in the area about the absorption of oligopeptides, even if further investigation is needed to clarify some of the aspects involved.

Another study used the combination of IVD and Ussing chamber to clarify the mechanisms related to the differences in the absorption rate of caseins and whey proteins (Mulet-Cabero et al., 2020b). For this purpose, the authors subjected varying casein:whey protein ratios to *in vitro* gastrointestinal digestion using a semi-dynamic gastric model, a static intestinal model, and an *ex vivo* absorption model (Ussing chamber). The authors commented that this methodological approach represented a powerful tool for understanding the mechanisms underlying the physiological impact of foods and for designing foods with different rates of nutrient digestion for the nutritional and health needs of different populations (Mulet-Cabero et al., 2020b). However, they also highlighted the limitations of these methods, such as the lack of fully accurate gastric emptying dynamics and the lack of gastric motility. Further limitations of the methods will be discussed later in this article.

To ascertain the effects of chestnut extracts (**CHEs**) on the digestibility of nutrients of different chemical compositions and how their metabolites affect intestinal integrity, we recently investigated the effect of CHE-derived metabolites on intestinal epithelial integrity (Tretola, 2021). The TEER was studied in porcine jejuna in the presence of three CHE-derived metabolites at three different dilutions or in their absence. To our knowledge, this was the first study coupling the INFOGEST harmonised *in vitro* digestion with the Ussing chamber system to directly investigate the effects of the phenolic metabolites obtained by digested CHE on intestinal epithelium integrity in pig jejunum. In that study, the hypothesis was that different concentrations of CHE-derived polyphenols differently affect intestinal epithelial barrier functionality. We found that polyphenols obtained by CHE *in vitro* digestion exert protective effects against external stressors on intestinal epithelial cell integrity *ex vivo* when used at low concentrations (Tretola, 2021).

The wide field of application of the combined use of INFOGEST and Ussing chamber is also demonstrated by studies focusing on the use of nanoparticles for nutritional interventions in humans such as studies on nanoscale systems developments (McClements and Li, 2010) or about the use of nanoparticles to increase the amount of fibre in diets (Mackie et al., 2019).

The combination of simulated digestion and the use of intestinal tissues in the perfusion chamber represents an opportunity to reproduce the effects of the predigested nanoparticles on the protective and complex layer of mucus, an environment that cannot be properly reproduced by traditional *in vitro* cell culture studies.

In the aforementioned study by Mackie and colleagues (2019), cellulose nanocrystals were exposed to products obtained by the small intestinal *in vitro* digestion and subsequently to the murine intestinal mucosa. Interestingly, the results showed that the cellulose nanocrystals were entrapped in the intestinal mucus layer and failed to reach the underlying epithelium, demonstrating the safety of these nanocrystals. A schematic representation of the INFOGEST protocol coupled with the Ussing chamber is reported in Fig. 2.

### *Advantages and limitations of the harmonised in vitro digestion method applied with Ussing chamber*

The INFOGEST *in vitro* digestion protocol and the Ussing chamber system offer, alone or in combination, a useful screening tool to investigate in depth how polyphenols can influence digestive processes or gastrointestinal physiology. However, the lack of simulated microbial activity represents the main limitation of the INFOGEST and Ussing chamber methods. It is known that dietary polyphenols impact the intestinal microbial composition (Tretola et al., 2019) but they also interact with microbial enzymes, leading to the production of a large number of polyphenol metabolites that can be absorbed or that can influence intestinal health or integrity (Hervert-Hernández and Goñi, 2011).

The abundance, and therefore the activity of the gut microbiota, is higher in the large intestine, where the competition for the undigested dietary compounds between bacteria and the host is low,

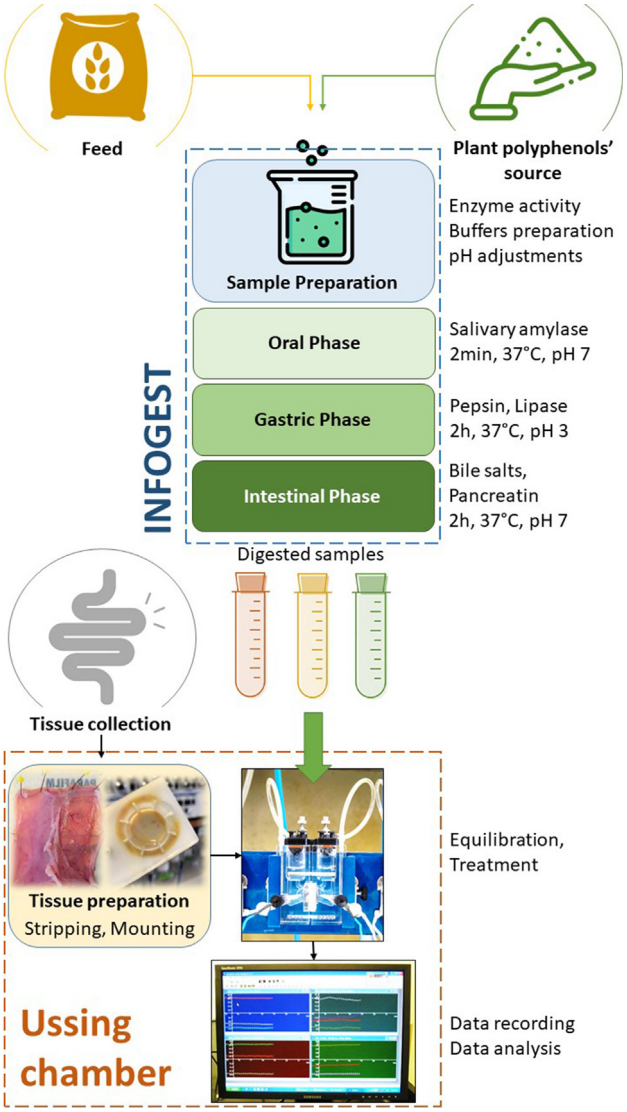


**Fig. 2.** Schematic representation of the INFOGEST protocol (source: Brodkorb et al., 2019) coupled with the Ussing chamber model in monogastrics.

the luminal pH is favourable for bacterial growth and the slower peristaltic movements allow bacteria to have more time to ferment the undigested substrates. Thus, if one is interested in the *IVD* of PP in the upper part of the digestive tract of monogastrics, or their effects on the integrity of the small intestine, the lack of simulated microbial activity could affect the outcome of the study to a lesser extent. However, this limitation cannot be neglected when studies are targeted at the large intestine or rumen. Methods such as the RUSITEC (Czerkawski and Breckenridge, 1977) to simulate *in vitro* bacterial activity exist, but this topic is not covered by the present review.

*Harmonised in vitro digestion method*

Despite its good intra- and inter-laboratory reproducibility, simplicity and low cost, the INFOGEST method has some limitations in mimicking the complex dynamics of digestive processes. For example, the pH is constant during the gastric phase, with no gradual addition of gastric fluids or gastric emptying. The enzyme activity in the different digestive phases is also constant, regardless of the type of food and its chemical composition.

The intestinal phase does not take into account the different segments (duodenum, jejunum, ileum) that differ for dilution, mineral content, pH, enzyme activities and microbial content making the method unsuitable for a detailed kinetic analysis. However, similarities have been found between the digestion endpoints between INFOGEST and *in vivo* data (Brodkorb et al., 2019), suggesting that the static INFOGEST model can be considered a valid alternative to *in vivo* methods only regarding the digestion endpoints but not the digestion kinetics. In studying the bioaccessibility of polyphenols, the model leads to their physiological release from the food matrix to the aqueous phase. However, it does not simulate the hydrolytic processes that usually take place along the brush border. Limitations are also linked to the lack of peristaltic movements that are needed to separate the bioaccessible phases. An extension, including the colonic phase fermentation, an essential step to bioactivate several phytochemicals, would further enhance the physiological appropriateness.

Concerning ruminants, the evaluation of forages or the bioaccessibility of bioactive compounds from feed is of great importance in ruminant nutrition research. However, we did not find any study that applied the INFOGEST model to digest *in vitro* the output of ruminal fermentation obtained either *in vivo* or *in vitro* to evaluate polyphenol bioaccessibility. An appealing but challenging approach would be to apply the INFOGEST intestinal digestion method to a previously fermented feed at the ruminal level. The long-term rumen simulation technique (Rusitec) is an example, capable of maintaining rumen-like fermentation over several days and it is widely used to mimic ruminal fermentation (Owen et al., 1991).

*Ussing chambers*

As the Ussing chamber technique uses intestinal tissue segments, the complexity of the intestinal morphology is taken into account during the interpretation of the obtained results (Westerhout et al., 2015). Furthermore, this method gives the possibility to investigate electrophysiological properties of the intestine in a specific GIT segment (e.g. duodenum, jejunum, ileum or cecum), which is not possible using single cell culture models.

One of the major limitations of the classic Ussing chamber is the low throughput due to the low number of tissues that can be tested at a time and leads to a low number of technical replicates within the same experiment. The small tissue size that is mounted on a slider (e.g. 1 $cm^2$ diameter for pigs) represents a small proportion of the entire gut, and this can lead to high variability between intestinal samples even when obtained from the same animal. To reduce the sample heterogeneity, a sufficient number of technical replicates needs to be considered. This limitation has been partially solved by the modified configuration of the system, which allows the simultaneous allocation of the tissues in up to 24 independent chambers. Moreover, to reduce the within-animal variability, it has been suggested to use contiguous samples.

Another major limitation of the method is its limited tissue viability. Under standard conditions, the viability of the tissues is assured for around 2 h. This period could be too short to investigate some of the aspects related to the impact of bioactive compounds on intestinal health, such as the production of inflammatory cytokines. Many Ussing chamber experiments are conducted in rodents where they may be sacrificed within a laboratory environment. However, farm animals are in general euthanised in a commercial or research abattoir where the Ussing chamber setup is usually not available. Because of the limited tissue viability, strategies to guarantee the optimal temperature and oxygenation of the tissues during the transport from the abattoir to the laboratory need to be adopted.

Finally, the preparation of the tissues for Ussing chambers requires highly hands-on experience skills. The removal of the seromuscular layer can be more or less difficult based on the kind of tissue and/or the species used, and the success in the proper mounting of the tissues in the chambers strongly depends on the operator. Therefore, experiments in native tissue are often difficult to complete successfully and are characterised by low throughput and the absence of nervous stimuli.

Especially when comparing the results of different studies, the method of euthanasia also needs to be taken into account as it can affect muscle contractility (Butler et al., 1990) and ultimately the Ussing chamber measurements.

The Ussing chamber can be applied to many different epithelial and endothelial tissues and is much more reliable than cell line models. In addition, it can be applied to cell cultures and organoid monolayers as well.

## Future perspectives on accessibility and effects in in vitro evaluation of phenolic compounds

Coupling *in vitro* fermentation models with *in vitro* intestinal digestion protocols (to further test the uptake and/or the effects of bioaccessible polyphenols directly on different intestinal segments by perfusion chambers) could allow the effects of phenolic compounds in feed to be investigated along with the entire GIT in ruminants by reducing experimental costs and the number of animals involved. However, this combined approach represents a great methodological and technical challenge, requiring considerable and heterogeneous expertise.

The Ussing chamber is a scientific tool that provides essential information to better understand transepithelial transport processes. However, few studies to date have used the Ussing chamber to clarify the intestinal uptake and the effects of accessible phenolic compounds on intestinal physiology. Studies focusing on the effects of polyphenols on the gastrointestinal barrier function and nutrient uptake in different species will help us better understand the effects of phenolic compounds on animal physiology.

The use of Ussing chamber coupled with harmonised *in vitro* digestion and, in ruminants, downstream to the *in vitro* ruminal and gastric simulated environments, could offer the possibility of obtaining results as close as possible and more relevant to the *in vivo* situations. This approach will advance our knowledge of the fate of PP along the entire gastrointestinal tract. In ruminants, we could obtain *in vitro* information about how phenolic compounds influence the ruminal ecosystem, which polyphenols will

be accessible into the intestine, how they influence intestinal enzyme activity, and their effect on nutrient uptake and epithelial integrity. The latter could be evaluated in a particular intestinal segment of interest that could belong to the small or large intestine. The starting concentration of the applied PP compound and metabolites must be carefully considered on the basis of existing knowledge in order to remain within a physiologically relevant context. This essential information, in combination with further metabolic and molecular examinations, will provide the basis for defining the optimal dietary strategies for a wide field of application in animal nutrition.

## Ethics approval

Not applicable.

## Data and model availability statement

None of the data were deposited in an official repository but are available upon request.

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## Declaration of interest

None.

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