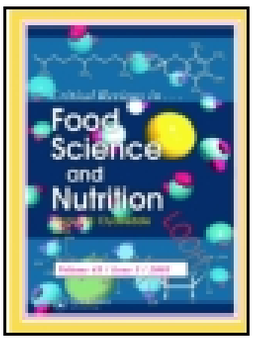

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REVIEW

Bringing the digestibility of prebiotics into focus: update of carbohydrate digestion models

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ABSTRACT

Oro-gastrointestinal digestion of dietary carbohydrates involves up to six different carbohydrases in a multistage process. Enzymes from the small intestinal brush border membrane play a major role in the digestibility of these substrates. However, to date, the inclusion of these small intestinal enzymes has been dismissed in most *in vitro* studies carried out, despite their importance in the degradation of carbohydrates. Several *in vitro* and *in vivo* studies have demonstrated the capability of brush border enzymes to degrade certain "non-digestible" carbohydrates to a different extent depending on their structural composition (monomeric composition, glycosidic linkage, etc.). In this sense, considering the available evidence, mucosal disaccharidases embedded in the small intestinal brush border membrane vesicles must be considered in addition to α-amylases; therefore, new approaches for the evaluation of the digestibility of carbohydrates have been recently reported. These new methods based on the utilization of the small intestinal enzymes present in the brush border membrane aim to fulfill the final and key step of the digestion of carbohydrates in the small intestine. Here, rat small intestinal extract enzymes as well as brush border membrane vesicles from pig have emerged as very reliable and useful tools to evaluate carbohydrate digestion. Thus, this review aims to go briefly through the most relevant digestion methods for carbohydrates that are currently available and to highlight the new improved methods, which include mammalian intestinal enzymes, and their current use in the evaluation of the digestibility of prebiotics.

KEYWORDS

Brush border membrane vesicles; carbohydrate digestion; mammalian intestinal enzymes; prebiotics; small intestinal digestion

Introduction

In the last decades, interest toward the modulation of the human microbiota has undergone huge growth due to its strong relationship with the regulation of health and well-being. To date, several illnesses have been related with the incorrect function or alterations in the composition of the human microbiota (Guinane and Cotter 2013; Jandhyala et al. 2015; Villanueva-Millán, Pérez-Matute, and Oteo 2015; Lerner, Neidhöfer, and Matthias 2017). One of the most commonly used approaches to regulate homeostasis, maintaining the proper behavior of microbiota, is the use of "prebiotics" which are selectively fermented in the gut, giving rise to positive changes not only in the singular ecosystem that inhabits the colon but also at a systemic level (Gibson et al. 2017). Thereby, since prebiotics were first defined, relevant advances have been made in the development of new prebiotic compounds. In this regard, one focus has pointed toward obtaining molecules with new structures that provide new and improved properties (Gullón et al. 2013; Díez-Municio et al. 2014; Míguez et al. 2016).

However, despite the great progress that has been made toward prebiotics and their benefits on human health, few studies have been made concerning changes to them during their passage through the upper gastrointestinal tract, even when structural differences could substantially affect their properties (Laparra et al. 2014; Li et al. 2015; Fernández et al. 2018; Larsen et al. 2019). The scarce information in this regard and the lack of specific methods to accurately determine their digestion, compel the use of general harmonized protocols such as the different AOAC or InfoGest methods, which could not reflect the whole range of enzymatic activities during the digestion of carbohydrates in the human small intestine.

The purpose of the present review is to compile the most important methods focused on carbohydrate digestion paying special attention to those based on the utilization of small intestinal extract from mammals in order to evaluate the potential partial digestion of prebiotics.

Resistance of prebiotic carbohydrates to the gastrointestinal digestion

One of the classical requirements for compounds to be considered as prebiotics is their resistance to upper gastrointestinal tract digestion. In this sense, ever since prebiotics were first defined in 1995, investigations have focused on their effect on the microbiota activity and/or composition.

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However, despite the generally accepted concept that prebiotics pass through the upper gastrointestinal tract without modifications, few efforts have been made toward the study of the resistance of these compounds to digestion and conditions in the upper gastrointestinal stage. It is important whether or not chemical or structural changes occur when they are exposed to this environment, because even minor differences could substantially affect their properties and, therefore, their impact on colonic microbiota (Laparra et al. 2014; Li et al. 2015; Fernández et al. 2018; Larsen et al. 2019).

Human oro-gastrointestinal degradation of carbohydrates constitutes a multistage process starting in the oral cavity, where the food is converted into a homogenous mass during mastication, with very scarce hydrolysis by the α-amylase present in the saliva. The main carbohydrate degradation and absorption occurs in the small intestine, being the main site for carbohydrate digestion involving the α-amylase secreted by the pancreas. In addition, brush border membranes of the intestinal mucosa contain several key enzymes for carbohydrate digestion present as multienzyme complexes, i.e., sucrase-isomaltase, lactase-phlorizin hydrolase, maltase-glucoamylase and trehalase being responsible for the final stage of luminal digestion (Figure 1) (Holmes and Lobley 1989; Hooton et al. 2015; Picariello, Ferranti, and Addeo 2016). Therefore, dietary carbohydrate digestion involves up to six different carbohydrases produced by three different organs (Table 1). Moreover, the mammalian upper gastrointestinal tract that contains a variety of distinct microbial populations could also affect the resistance of these substrates. In this sense, the small intestine, which has more acidic conditions and higher levels of oxygen and antimicrobials than the colon, is dominated by fast-growing facultative anaerobes, such as Lactobacillaceae or Enterobacteraceae, that tolerate the combined effect of antimicrobials and bile salts (Donaldson, Lee, and Mazmanian 2016). Moreover, conditions such as the presence of oxygen, antimicrobial compounds (bile salts) or pH limit the bacterial density below 104 colony-forming units (cfu/g) and only at the distal end of the small intestine, in the terminal ileum, bacterial densities reach levels similar to those found in the large intestine. With all these characteristics, it is not misplaced to consider that prebiotic compounds could suffer changes that could affect their properties when they reach the colon to be fermented.

Models for the assessment of digestibility and standardized protocols

To date, only scarce and fragmented information on their passage through the small intestine is available. As is well-known, *in vivo* feeding methods, using animals or humans, usually provide the most accurate results. In this sense, few *in vivo* studies have provided evidence of the partial degradation of prebiotic compounds after their passage through the small intestine. Holloway, Tasman-Jones, and Maher (1983) pointed out a recovery of 68% of pectin at the human terminal ileum in an ileostomy study, whereas Saito et al. (2005) observed a recovery rate of 90% in the terminal ileum after colonic intubation of volunteers. Moreover, Hernández-Hernández et al. (2012) found 13-53% of small intestinal digestibility of purified galactooligosaccharides (GOS, with a degree of polymerization from 3 onwards) in an *in vivo* study with rats. However, these methods have several drawbacks, such as high costs, ethical constraints, limitations in sampling from the small intestine, and the fact that they are time consuming, which explains why much effort has been devoted to the development of *in vitro* procedures. *In vitro* digestion models may provide a very useful alternative to animal and human models by rapidly screening food ingredients. In this sense, the most frequently used approach to measure the digestion process is by continuous or static simulation of human physiological conditions as much as possible, taking into account the presence of digestive enzymes and their concentrations, pH, temperature, digestion time, and salt concentrations, among other factors (Table 2) (Kopf-Bolanz et al. 2012; Minekus et al. 2014). Very recently, Hernández-Hernández (2019) carried out an overview of different methodologies used for the assessment of the digestibility of carbohydrates.

Continuous in vitro digestion methods

Although the majority of models reported in the literature are static simulators, some computer-controlled multi-compartmental continuous system models can overcome some limitations present in static models allowing the simulation of dynamic aspects of digestion, such as the transport of digested components, variable enzyme concentrations, pH changes, peristaltic movements, continuous changes, and secretion flow rates (Ouwehand and Vaughan 2006). To date, these multi-compartmental models that mimic the different gastrointestinal stages in a continuous flow represent the most advanced attempt at simulating interdependent physiological functions within the stomach lumen, small intestine and human gut (Molly, Vande Woestyne, and Verstraete 1993; Minekus et al. 1995; Barroso et al. 2015; Dupont et al. 2019). However, these models require highly complex and substantial hardware and software and are still expensive to set up and maintain in a regular laboratory; therefore, static models have an economic and large throughput advantage.

Ferreira-Lazarte, Moreno, et al. (2019) studied the digestibility and fermentability of citrus pectin in a Dynamic Gastrointestinal Simulator (simgi®) and observed a 12% loss of pectin during its passage through the stomach and the small intestine after the evaluation of the remaining polymer by HPLC-ELSD analysis. Some authors attributed the loss of pectin during digestion to the effect of the bacteria that can be present in the digestive tract (Saito et al. 2005). As in the prototype of Ferreira-Lazarte, Gallego-Lobillo, et al. (2019), bacteria are only confined in the colon compartments, and the modifications observed in the structure of pectin could be attributed to the chemical effects of bile salts and pancreatic fluids.

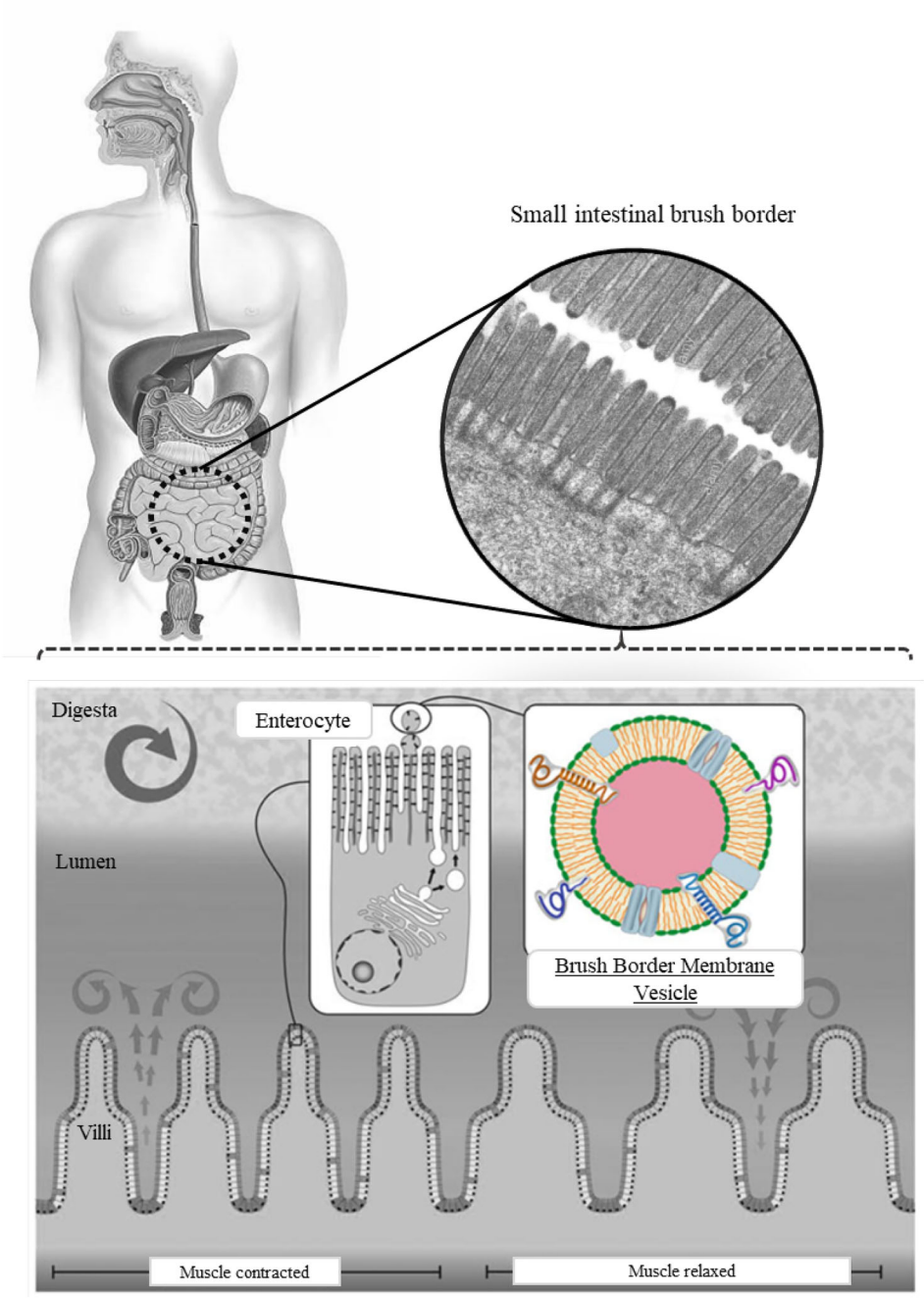

Figure 1. Brush border membrane enzymes presence and formation of vesicles in the small intestine. Colored proteins inside the membrane bilayer (green and yellow), represent the brush border enzymes with peptide (gold and pale blue) and glycosylphosphatidyl-inositol (dark blue and purple) anchors. BBMVs can be released into the adjacent periapical space, however, it is likely that the mucus layer overlying the ephitelia retards the egress of BBMVs from the periapical space into the lumen so that a significant amount of BBMVs would remain in close proximity to the intestinal mucosa.

Furthermore, Bellmann et al. (2018) presented a dynamic digestion model focused on carbohydrates, called TIMcarbo, which was based on the TNO GastroIntestinal Model (TIM) (Minekus et al. 1995). This model mimics carbohydrate uptake by the epithelium through dialysis of the products of digestion by a commercial rat small intestinal extract enzyme and the addition of a bacterial lactase given the low activity found in these extracts. Therefore, TIMcarbo would represent a highly relevant tool to evaluate the digestion of whole foods and meals where the physical changes of the digesta (e.g., viscosity or particle size reduction) are key determinants of the rate of carbohydrate digestion and absorption *in vivo* (Lifschitz, Grusak, and Butte 2002; Turnbull, Baxter, and Johnson 2005). However, this modified method is not so suitable for screening studies or for the evaluation of low available amounts of carbohydrates, which is typically a limiting factor when the digestibility of novel carbohydrates produced at laboratory scale needs to be assessed (Hernández-Hernández et al. 2019). In this sense, static *in vitro* methods can constitute a good alternative.

Table 1. Human carbohydrases involved in dietary carbohydrate digestion.

Digestive carbohydrases	Type of enzyme	Glycoside hydrolase family[a]	Production organ/main site of digestion	Glycosidic linkage specificity	Main substrates[b]	Main products[b]
Salivary α-amylase[c]	Secreted (α-glucosidase)	13	Salivary gland/mouth	Glcα(1→4)Glc	Starch; linear maltooligosaccharides (n > 6)	Maltose; maltotriose; α-dextrins
Pancreatic α-amylase[c]	Secreted (α-glucosidase)	13	Pancreas/small intestine	Glcα(1→4)Glc	Starch; linear maltooligosaccharides (n > 6)	Maltose; maltotriose; α-dextrins
Sucrase-isomaltase	Mucosal (α-glucosidase)	31	Small intestine (brush border membrane)/ small intestine	Glcα(1↔2)βFru Glcα(1→4)Glc Glcα(1→6)Glc	Sucrose; isomaltose; maltose; maltotriose; α-dextrins	Glucose; fructose
Maltase-glucoamylase	Mucosal (α-glucosidase)	31	Small intestine (brush border membrane)/ small intestine	Glcα(1→4)Glc Glcα(1→6)Glc	Linear and branched maltooligosaccharides (n = 2–9)	Glucose
Lactase-phlorizin hydrolase	Mucosal (α-glycosidase)	1	Small intestine (brush border membrane)/ small intestine	Glcα(1→4)Gal Glcα(1→4)Glc	Lactose, cellobiose, cellotriose, cellulose	Glucose; galactose
Trehalase	Mucosal (α-glucosidase)	37	Small intestine (brush border membrane)/ small intestine	Glcα(1↔1)αGlc	Trehalose	Glucose

Reproduced from Hernández-Hernández et al. (2019).
[a]According to CAZy database (http://www.cazy.org/; Lombard et al. 2014).
[b]Based on and updated from Alpers (2003).
[c]Human salivary and pancreatic amylases have 94% amino acid identity although they are encoded by different genes (Meisenberg and Simmons 2016).

Table 2. Overview on the *in vitro* intestinal digestion studies based on simulated fluids (without mucosal enzymes) for carbohydrates.

Carbohydrates evaluated	Digestion model	Digestion and fermentation stages/conditions	Degradation	References
Fructooligosaccharides	Static (InfoGest)	Oral, gastric and small intestinal digestion (pancreatin and porcine bile extract)	Slight degradation in GF_3	Nobre et al. (2018)
Hydrolyzed Curdlan ((1→3)-*β*-D-glucan oligosaccharides)	Static (InfoGest)	Oral, gastric and small intestinal digestion (pancreatin and porcine bile extract)	No	Shi et al. (2018)
Fructooligosaccharides	Dynamic (SHIME)	Gastric, small intestinal digestion (pancreatin and bile salts) and colon (ascending transverse and descending)	No reported	Sivieri et al. (2014)
Citrus Pectin	Dynamic (simgi®)	Gastric, small intestinal digestion (pancreatin and bile salts) and colon (ascending, transverse and descending)	Slight Mw decrease after intestinal digestion	Ferreira-Lazarte, Moreno, et al. (2019)
Galactose rich oligosaccharides from Rhamnogalacturonan-I	Dynamic (TIM-1)	Gastric and Small Intestine (duodenum, jejunum, ileum - pancreatin, bile salts and dialysis)	∼ 80% resistance	Khodaei et al. (2016)
Polysaccharide from *Hericium ernaceus* ((1→6)-α-D-galactopyranosyl backbone)	Static	Gastric, and small intestinal digestion (pancreatin and bile salts)	Mw decrease and free monosaccharides increase	Yang et al. (2018)
Polysaccharides from *Gracilaria rubra*	Static	Oral, gastric and small intestinal digestion (pancreatin and porcine bile extract)	No	Di et al. (2018)
Polysaccharides from *Rosa roxburguii*	Static	Oral, gastric and small intestinal digestion (pancreatin and porcine bile extract)	Slight Mw decrease after stomach and intestinal digestion	Wang, Li, et al. (2019)
Polysaccharides from Fuzhuan brick tea	Static	Oral, gastric and small intestinal digestion (pancreatin and porcine bile extract)	No	Chen, Xie, et al. (2018)
Polysaccharides from brown seaweed (*Ascophyllum nodosum*) and *Coralline pilulifera*	Static	Oral, gastric and small intestinal digestion (pancreatin and porcine bile extract)	No	Chen, Xu, et al. (2018) Wang, Chen, et al. (2019)
Polysaccharides from *Aloe vera*	Static	Small intestinal digestion (α-amylase)	No	Tornero-Martínez et al. (2019)
Polysaccharides from *Aspergillus cristatus*	Static	Oral, gastric and small intestinal digestion (pancreatin and porcine bile extract)	No	Rui et al. (2019)
Polysaccharides from *Gracilaria lemaneiformis*	Static	Oral, gastric and small intestinal digestion (pancreatin and porcine bile extract)	No	Han et al. (2020)

Static in vitro digestion methodologies

Static digestion methods represent the simplest techniques and can include two or three separate digestion steps (oral, gastric and intestinal). The oral phase is sometimes not taken into account given that the process in the mouth lasts from a few seconds to minutes, and since the salivary pH value is close to neutral, the degradation of significant compounds from food samples is not expected in this stage. To date, most *in vitro* techniques for the analysis of the glycaemic properties of foods have their roots in the earlier methods for the analysis of dietary fiber, total carbohydrates and resistant starch in foods (Woolnough et al. 2008). In this sense, current methods are based on those proposed by Southgate (1969), Englyst, Wiggins, and Cummings (1982), and/or Jenkins et al. (1982), where readily digestible starch was hydrolyzed with α-amylase and pullulanase, and resistant starch was subjected to more exhaustive hydrolysis with amyloglucosidase. Since then to the present day an extensive number of different methods for the digestibility of carbohydrates have been reported. In this regard, the most widely accepted standardized *in vitro* method for digestion was developed in 2014 as an attempt to unify the different methodologies used until that moment and to produce data that are more comparable and reproducible between studies (Minekus et al. 2014; Egger et al. 2016; Brodkorb et al. 2019). The static protocol published by the InfoGest network represents an international consensus of scientists from 32 countries working in the field of digestion, and it has been widely used and supported in several works since its release (Egger et al. 2016, 2017; Bohn et al. 2018; Verhoeckx et al. 2019).

Concerning small intestinal digestion, this method presents two approaches regarding the enzymes used at this stage (Minekus et al. 2014). Firstly, the use of a pancreatic extract (pancreatin) containing all the relevant enzymes is suggested for reasons of simplicity. As an alternative, individual enzymes, such as trypsin, chymotrypsin, pancreatin lipase, colipase, pancreatin amylase can also be used. However, despite the clear usefulness of this method for the evaluation of the digestibility of proteins, lipids and starchy carbohydrates, the complexity of the gastrointestinal process in the small intestine means that the enzymes proposed in this method for intestinal digestion cannot reflect the broad array of enzymatic activities of the human gut toward carbohydrates since small intestinal mucosal carbohydrases are

Table 3. Overview on the *in vitro* intestinal digestion studies based on the mammalian mucosal small intestinal enzymes (rat and pig) for carbohydrates.

Carbohydrates evaluated	Digestion model	Digestion and fermentation stages/conditions	Degradation	References
Isomalto/Malto-polysaccharides	Static	Small Intestine: Brush Border carbohydrases from rat intestinal acetone powder	Yes	Leemhuis et al. (2014)
GOS from lactose GOS from lactulose Lactulose Fructooligosaccharides Lactosucrose	Static	Small Intestine: Brush Border carbohydrases from rat intestinal acetone powder	Yes – structural differences determined the resistance to degradation	Ferreira-Lazarte, Olano, et al. (2017)
Lactose and Lactulose	Static	Small Intestine: Brush Border carbohydrases from rat intestinal acetone powder	Yes – structural differences determined the resistance to degradation	Gallego-Lobillo, Ferreira-Lazarte, Hernández-Hernández, and Villamiel (2020)
Fructans and α-galactooligosaccharides	Static	Small Intestine: Brush Border carbohydrases from rat intestinal acetone powder	Yes – structural differences determined the resistance to degradation	Gallego-Lobillo, Ferreira-Lazarte, Hernández-Hernández, Montilla et al. (2020)
Isomaltooligosaccharides	Static	Enzyme mixture (pancreatin, invertase and amyloglucosidase) and brush border carbohydrases from rat intestinal acetone powder	Yes	Hu et al. (2017)
Maltose/Sucrose isomers	Static	Small Intestine: Brush Border α-glucosidases from rat intestinal acetone powder	Yes	Lee et al. (2016)
Starch samples	Static	Oral, gastric and small intestinal (pancreatin, bile salts and brush border carbohydrases from rat intestinal acetone powder)	Yes	Garcia-Campayo et al. (2018)
GOS from lactose GOS from lactulose	Static (InfoGest modified)	Oral, gastric and small intestinal (pancreatin, bile salts and brush border carbohydrases from rat intestinal acetone powder)	Yes – milk matrix did not modify their digestibility	Ferreira-Lazarte, Montilla, et al. (2017)
Turanose and resistant starch	Static (InfoGest modified)	Oral, gastric and small intestinal (pancreatin, bile salts and brush border carbohydrases from rat intestinal acetone powder)	Yes	Park, Chandrasekaran, and Yoo (2019)
Starchy and fiber commercial food products	Dynamic (TIMcarbo)	Oral, gastric and small intestinal (pancreatin, bile salts) and brush border enzyme digestion (rat brush border enzyme extract and commercial lactase)	Yes	Bellmann et al. (2018)
GOS from lactose GOS from lactose	Static	Small Intestine: brush border membrane vesicles from pig	Yes – $\beta(1\rightarrow3)$ linkages more hydrolyzed	Ferreira-Lazarte, Gallego-Lobillo, et al. (2019)
GOS from lactose	Static	Small Intestine: brush border membrane vesicles from pig	Slight GOS degradation	Tanabe, Nakamura, and Oku (2014) and Tanabe et al. (2015)
Sialyllactoses	Static (InfoGest modified)	Oral, Gastric, and Small Intestine (pancreatin, bile salts and brush border membrane vesicles from pig)	Yes	Moon et al. (2016)

not regularly taken into account (Table 1). There is therefore the lack of a prescribed final step during intestinal digestion in which disaccharidases from intestinal mucosa would complete carbohydrate digestion (Goodman 2010).

Studies on the digestion of different types of fiber such as resistant starch, FOS, pectic polysaccharides from kiwifruit, and sugar beet pectin using simulated gastric and intestinal fluids similar to the InfoGest method, have shown a partial degradation or changes in their structural features (Carnachan et al. 2012; Logan, Wright, and Goff 2015; Foucault et al. 2016; Sancho et al. 2017). Molecular weight diminution and decreases in their initial concentration were observed in these substrates, although the enzymes (pancreatin, α-amyloglucosidase or invertase) and concentration used were different in each study, not showing any consensus between methods.

The Association of Official Analytical Chemists (AOAC) developed an integrated determination method for dietary fiber, including non-digestible oligosaccharides (NDOs) and resistant starch (AOAC 2009.01) (McCleary et al. 2010), which was later modified in 2015 (McCleary, Sloane, and Draga 2015). This method, as well as the others, is based on the use of isolated digestive enzymes. Porcine pancreatic α-amylase, mucosal α-glucosidase and a fungal amyloglucosidase from *Aspergillus niger* are used to produce the complete hydrolysis of digestible saccharides and, therefore, to distinguish between digestible and non-digestible carbohydrates. However, these enzymes cannot completely hydrolyze

digestible saccharides such as sucrose, lactose, panose, etc. since they do not represent the fully complex enzymatic environment of the small intestine. As a result, digestible saccharides are not fully degraded and are miscategorized as non-digestible carbohydrates leading to an inaccurate determination of these resistant carbohydrates (Tanabe, Nakamura, and Oku 2014).

New methods based on mammalian small intestine extracts for carbohydrate digestion

The scarce information about the use of mammalian intestinal enzymes has constituted a necessity for some authors in developing methods that may provide a better understanding of the processes occurring in the small intestine, so that it can be used in the evaluation of this functionality in foods.

In addition to the inclusion of intestinal brush border enzymes in the TIMcarbo, alternative methods based on the use of mammalian intestinal extracts, which could provide a more accurate approach of the intestinal process, have also been presented as a good option to overcome the disadvantages of the use of isolated enzymes (Table 3). In this sense, similarity between humans and rats with regard to the hydrolyzing activity of small intestinal disaccharidases was proved (Oku et al. 2011). Small intestinal mucosa obtained from healthy human donors and rats were used to test the digestibility of different oligosaccharides showing a similar enzymatic activity, providing a good alternative to the evaluation of functional food digestion in the small intestine.

Methods based on rat small intestinal extract

To date, there are limited reports regarding the use of a rat small intestinal extract for the evaluation of the digestibility of non-digestible carbohydrates. The most common use of these extracts refers to previous steps of digestion focusing on their fermentability (Ito et al. 2008; Kaulpiboon et al. 2015). However, other studies have also successfully applied these intestinal enzymes from rats for the assessment of the digestibility of resistant carbohydrates, such as fructooligosaccharides (FOS) (Oku, Tokunaga, and Hosoya 1984), GOS (Ohtsuka et al. 1990), as well as to a range of maltose and sucrose isomers/polymers (Lee et al. 2016; Shin et al. 2019).

Ferreira-Lazarte, Olano, et al. (2017) reported a new *in vitro* method to evaluate the digestibility of dietary oligosaccharides under physiological conditions using extracts derived from the small intestine of rats (RSIE) with the advantage of requiring small amounts of carbohydrate substrates to be assessed. The proposed method also included a reliable analytical method, such as GC-FID, where remaining carbohydrates as well as the products of digestion can be evaluated identifying the specific structural changes during digestion (type of glycosidic linkage hydrolyzed, monosaccharides released, etc.). This method represents a useful, simple and cost-effective tool to distinguish between digestible and non-digestible carbohydrates. The similarity found between rat intestinal disaccharidase activities (maltase, sucrase, palatinase, trehalase and lactase) and their equivalent in humans (Oku et al. 2011), as well as the good correlation found between this method and data collected *in vivo* (Molis et al. 1996; Hernández-Hernández et al. 2012; Jantscher-Krenn, Marx, and Bode 2013), endorse the suitability of these mammalian extracts to assess the digestibility of dietary carbohydrates. The digestion rate of recognized and potential prebiotics (GOS, FOS, lactulose, novel lactulose-derived galactooligosaccharides (OsLu) and lactosucrose) was assessed and compared with this method. Prebiotic and potential prebiotic compounds were highly resistant to small intestine enzymes; however, structural differences exhibited an important effect on their susceptibility to degradation. Remarkably, different predominant linkages in GOS demonstrated a key role in resistance to degradation. For instance, $\beta(1\rightarrow6)$ linkages in GOS mixtures showed significantly higher resistance when compared to $\beta(1\rightarrow4)$ linkages, which were the most susceptible to degradation in all prebiotic samples tested. Monomeric composition was also a critical factor for digestibility, thus, oligosaccharides with a terminal fructose (those derived from lactulose) were less prone to hydrolysis than those with glucose (GOS). Lactulose and FOS resistance was higher as compared to GOS and OsLu, due to their monomer composition and resistant linkages. The molecular weight of OsLu, which has been related to slower fermentation by the colonic microbiota than lactulose, being able to reach the distal colon without major alterations (Cardelle-Cobas et al. 2011, 2012), seemed to also provide higher resistance to intestinal enzymes. In this sense, the trisaccharide fraction (mainly β-Gal-(1→6)-β-Gal-(1→4)-Fru) present in OsLu was almost not degraded as compared to lactulose after *in vitro* digestion, whereas the trisaccharide fraction in GOS, mainly β-Gal-(1→4)-β-Gal-(1→4)-Glc and β-Gal-(1→6)-β-Gal-(1→4)-Glc, was slightly more prone to degradation. The relationship between the structure of GOS and resistance to mammalian intestinal enzymes and their bioactive effect was already pointed out in previous reports (Rastall et al. 2005; Hernández-Hernández et al. 2012), however, no comparison of the digestibility of different types of GOS from lactose and lactulose using a mammalian intestinal extract was carried out before. Furthermore, a recent study carried out by Gallego-Lobillo, Ferreira-Lazarte, Hernández-Hernández, and Villamiel (2020), which evaluated the kinetics of degradation of digestible and non-digestible saccharides (lactose and lactulose, respectively) with RSIE under physiological conditions, showed the higher resistance of galactosyl-fructose linkages (with lower hydrolysis rate constants (Km) and higher $t_{1/2}$), whereas lactose was highly degraded by these enzymes (higher Km and lower $t_{1/2}$), supporting, therefore, the suitability of the use of RSIE for the simulation of carbohydrate digestion.

In addition, these small intestinal enzymes were also used to assess their impact on different types of functional fiber such as several types of fructans and α-galactooligosaccharides (Gallego-Lobillo, Ferreira-Lazarte, Hernández-Hernández, Montilla et al. 2020). In line with previous studies, inulin-type fructans and fructooligosaccharides were

found to be very resistant to digestion. Strikingly, the degree of hydrolysis of non-fructosylated α-galactooligosaccharides derived from peas was around 60%. Factors as Mw and type of linkage contributed to the resistance of fructans to digestion, whereas α-GOS were hydrolyzed considerably in a similar way to the degradation of β-GOS derived from lactose and lactulose observed in previous works.

Moreover, given that the mixtures of prebiotics are usually included in different foodstuffs, mainly milk and dairy products, there is the need to consider the effect of the food matrix on prebiotic digestibility. Therefore, Ferreira-Lazarte, Montilla, et al. (2017) reported the utilization of mammalian intestinal enzymes in a synergistic manner with the current standardized method (InfoGest) to evaluate the *in vitro* gastrointestinal digestion of prebiotics added to milk. The use of pancreatic fluids and bile salts to simulate intestinal digestion has provided evidence of the limitation of these models for carbohydrate digestion since hardly any modifications were observed in the carbohydrate fraction of the samples. Conversely, the utilization of the RSIE to digest samples after their gastric stage demonstrated its suitability to hydrolyze carbohydrates showing measurable decreases in this fraction. Firstly, higher amounts of lactose did not seem to affect prebiotic degradation after 2 hours of digestion with the RSIE. Degradation found in the oligosaccharide fraction of GOS (35%) and OsLu (15%) within the milk was similar to that obtained by Ferreira-Lazarte, Olano, et al. (2017), that is 34 and 18%, respectively. High decreases of lactose content were observed whereas lactulose remained as the least prone to disaccharide degradation. In line with the results of Ferreira-Lazarte, Olano, et al. (2017), oligosaccharides from the predominant β(1→4)-GOS mixture exhibited the highest degradation after digestion, whereas oligosaccharides from β(1→6)-OsLu stood as the most resistant structure with 85% of composition intact after 2 hours of digestion, highlighting the suitability of the inclusion of these substrates within a real food context.

Therefore, in a robust digestion method for carbohydrates the inclusion of a step using mammalian small intestinal extract is needed to fully cover the carbohydrase activities of the gastrointestinal system. In this sense, rat intestinal enzymes could provide a useful and reliable tool to determine the digestibility of dietary carbohydrates in static isolated models (Ferreira-Lazarte, Olano, et al. 2017; Shin et al. 2019) as well as a part of a complete gastrointestinal digestion model (Ferreira-Lazarte, Montilla, et al. 2017; Garcia-Campayo et al. 2018). However, despite the positive results obtained, the small size of these animals could create a challenge for their use as human models due to the very different anatomy and physiology as compared to humans.

Methods based on pig small intestinal enzymes

Pig has emerged as an important model due to their anatomical and physiological similarity to humans and the human genome (Humphray et al. 2007), as well as their larger litter size, and the fact that they are a food source may help avoid ethical concerns (Kuzmuk and Schook 2011). The use of pigs has proved to be a robust model for several studies such as tissue engineering, imaging, surgery, chemotherapy, radiation studies, cancer, atherosclerosis, myocardial infarction, and general cardiovascular models, which cannot be carried out accurately or have failed in small animals (Kuzmuk 2009; Jensen et al. 2010; Schook et al. 2015). The physiological similarity between humans and pigs in terms of digestive processes places the pig as a robust model for human digestive and colonic studies (Heinritz, Mosenthin, and Weiss 2013). Thus, the use of small intestinal materials from pigs could represent a more reliable method as well as a step forward toward the development of a robust method that is comparable to humans in order to gather information about dietary carbohydrate digestion. Nonetheless, the use of these extracts has still been sparsely used even though they have proved their successful utility on carbohydrate digestion and are similar to human intestinal activity (Lander et al. 2001; Humphray et al. 2007; Oku et al. 2011).

Alternative methods based on the use of mammalian intestinal enzymes derived from pigs (Tanabe, Nakamura, and Oku 2014) or weaning piglets (Strube et al. 2015) have been proposed. Given the limitations of the AOAC 2009.01 method, an improvement method by the addition of mammalian small intestinal enzymes from pigs was suggested (Tanabe, Nakamura, and Oku 2014). In this sense, the incomplete degradation of digestible saccharides that leads to overestimating NDOs was improved by the addition of the enzymes present at the small intestinal brush border from pigs, providing, therefore, a more realistic environment and an accurate quantification method of NDOs in processed food (Tanabe et al. 2015).

The application of intestinal disaccharidases, that are identified as glycoproteins linked to the apical membrane and are actively budded off as brush border membrane vesicles (BBMVs) (Figure 1) (McConnell et al. 2009; Hooton et al. 2015), from pig has recently been shown to be a reliable and useful approach to evaluate prebiotic carbohydrate digestibility, under physiological conditions of pH, temperature and time. Isolated BBMVs from the intestinal mucosa were used by Ferreira-Lazarte, Gallego-Lobillo, et al. (2019) to evaluate the digestibility of prebiotic oligosaccharides (GOS and OsLu). Regarding enzymatic activity, BBMVs showed higher values when compared to a commercial intestinal extract from rats mainly due to the exhaustive fractionation and purification carried out in the obtainment of the membrane vesicles. Digestibility assays, in line with previous reports (Ferreira-Lazarte, Montilla, et al. 2017; Ferreira-Lazarte, Olano, et al. 2017), confirmed the resistance of β(1→6) linkages as compared to β(1→4) linkages. Moreover, predominantly β(1→3) linkages in GOS that were also analyzed in this study, revealed the high susceptibility of these structures to intestinal disaccharidases with a degradation of 44%, whereas β(1→4) and β(1→6) showed a hydrolysis of 23 and 12%, respectively, after 2 hours of digestion.

Likewise, another recent study on the obtainment of GOS using the trans-β-galactosylation activity of the pig

β-galactosidase embedded in the BBMVs has revealed the high preferable synthesis of GOS linked by $\beta(1\rightarrow3)$ as compared to those linked by $\beta(1\rightarrow4)$ or $\beta(1\rightarrow6)$ (Julio-Gonzalez et al. 2019). In good agreement with this, the β-Gal-(1→3)-Gal- glycosidic bond was also identified, together with the β-Gal-(1 →5)-Fru linkage, as being preferentially digested when lactulose was incubated with pig BBMVs (Julio-Gonzalez et al. 2020). Therefore, considering that enzymes can catalyze reversible reactions in either direction (Abdul Manas, Md Illias, and Mahadi 2018), data obtained by these authors support the hypothesis that most glycosidic linkages formed when intestinal β-galactosidase acts as transgalactosidase, are preferentially broken under hydrolytic conditions. In addition, specific monosaccharide composition provided a high resistance on several structures such as galactosyl-galactoses, galactosyl-fructoses and, especially, on the trisaccharide of OsLu ($\beta(1\rightarrow6)$ (only 9.8% of hydrolysis was reported after 2 hours), highlighting the key role of these structural features.

Conclusions

The lack of thorough studies on the intestinal degradation of non-digestible carbohydrates has led to the use of standardized official methods to determine their digestibility despite their limitation (McCleary et al. 2010; Egger et al. 2016; Drechsler and Bornhorst 2018). To solve this problem, important efforts have been made to develop an adequate *in vitro* model for carbohydrates, the intestinal mucosal enzymes being used only in few methods that have been recently developed (Ferreira-Lazarte, Olano, et al. 2017; Garcia-Campayo et al. 2018; Shin et al. 2019).

Evaluation of the digestibility of carbohydrates with new emerging methods based on the use of mammalian intestinal enzymes have revealed essential structure-function relationships in prebiotic carbohydrates, such as the strong resistance of $\beta(1\rightarrow6)$ oligosaccharides, especially of those derived from lactulose, which have been shown to be the most resistant GOS to *in vitro* digestion. Thus, when aiming for the potential development of tailored and new generation prebiotics, oligosaccharides derived from lactulose would represent the ideal candidates to be taken into account, given also the large amount of studies supporting their technological and biological properties (Villamiel et al. 2014; López-Sanz et al. 2015, 2018; Barroso et al. 2016; Fernández et al. 2018).

The *in vitro* digestion models based on the utilization of rat small intestinal extract proved to be a useful, reliable and efficient approach to evaluating the digestibility of dietary carbohydrates (digestible and non-digestible), overcoming the limitations of the current standardized methods for *in vitro* gastrointestinal digestion. Moreover, the utilization of a model based on small intestinal BBMVs from pigs would be a more realistic approach to study the influence of glycosidic linkages, degree of polymerization and monomeric composition on resistance to intestinal digestion of selected prebiotics. Thus, although there is still work to be done in regard to developing more standardized conditions concerning enzymatic activities, enzyme/substrate ratios or a regular supply of pig mucosal intestinal enzymes, the use of these enzyme material, combined with the standardized methodologies, clearly involves the next step toward a final and complete small intestinal digestion simulation for dietary carbohydrates.

Lastly, the prebiotic concept maintains that these substrates should be "non-digested" by intestinal enzymes, reaching the colon at least almost intact; however, findings obtained so far with current studies have revealed the partial degradation of some of these substrates. Therefore, the studies that have been discussed in the present review indicate that certain prebiotic oligosaccharides may not reach the colon fully intact, thus challenging the general belief about their complete non-digestibility, and, although more studies are required to focus, for instance, on the role of the food matrix or on the better understanding of the physiologically relevant activity of BBMVs, a possible revision of the current concept of the digestibility of prebiotics could be envisaged.

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