UNIVERSIDAD AUTÓNOMA DE MADRID

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Departamento de Química-Física Aplicada

In vitro digestibility and fermentability of selected prebiotics and functional carbohydrates with prebiotic potential

ALVARO FERREIRA LAZARTE

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ALVARO FERREIRA LAZARTE

Thesis submitted in fulfilment of the requirements for the degree of doctor at Autónoma University of Madrid (UAM)
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CERTIFY:

That **Alvaro Ferreira Lazarte,** has performed, under their supervision, the research work entitled**: "*In vitro* digestibility and fermentability of selected prebiotics and functional carbohydrates with prebiotic potential."** This work memory is submitted in fulfilment of the requirements for the degree of doctor at Autónoma University of Madrid (UAM).

Madrid, 27 de marzo de 2019

Fdo. Dr. María del Mar Vilamiel Guerra

Fdo. Dr. F. Javier Moreno Andújar

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Contents

ABBREVIATION LIST

Abbreviation	Explanation
CNS	Central nervous system
SCFA	Short chain fatty acid
FAO	Food and Agricultural Organization
ISAPP	International Scientific Association for Probiotics and Prebiotics
NDO	Non-digestible Oligosaccharide
FOS	Fructooligosaccharides
SMP	Skim Milk Powder
GOS	Galactooligosaccharides
HMOs	Human Milk Oligosaccharides
OsLu	Oligosaccharides derived from lactulose
IMOS	Isomaltooligosaccharides
XOS	Xilooligosaccharides
GalA	Galacturonic acid
HG	Homogalacturonan
RG-I	Rhamnogalacturonan-I
RG-II	Rhamnogalacturonan-II
DM	Degree of methoxyl esterification
HM	High methoxyl
LM	Low methoxyl
MP	Modified pectin

POS	Pectic oligosaccharides
o-NPG	*o*-nitrophenyl-*β*-D-galactopyranoside
p-NPG	*p*-nitrophenyl-α-glucopyranoside
BBMV	Brush Border Membrane Vesicles
RSIE	Rat Small Intestine Extract
GC-FID	Gas Chromatograpy – Flame Ionization Detector
ELSD	Evaporative Light Scattering Detector
TMSO	Trimethyl silylated oximes
IS	Internal Standard
TFA	Trifluoroacetic Acid
HMDS	Hexamethydisilazane
MTBSTFA	*N-(tert*-butyldimethylsilyl)-N-methyltrifluoroacetamide
Simgi®	SIMulator Gastro-Intestinal model
ST	Stomach
SI	Small Intestine
AC	Ascending Colon
TC	Transverse Colon
DC	Descending Colon

SUMMARY - RESUMEN

Summary

The regulation of microbiota through the use of prebiotics has been widely studied in the last decades, due to its high correlation with human health and several diseases. In this sense, there are a huge number of reports regarding the beneficial effects of these compounds on gut microorganisms, however, scarce efforts have been made towards their previous passage through gastrointestinal digestion. In a first part of this PhD Thesis, the digestibility of several recognised prebiotics has been studied and results are presented in the first three chapters. A more efficient and reliable *in vitro* method based on the use of rat small intestinal extract has been proposed for carbohydrate digestion overcoming the limitations of general standardised models for gastrointestinal digestion which overlook the critical role of the small intestine mucosal carbohydrases. Likewise, considering the high physiological and anatomical similarity of the pig and human digestive tracts, the isolation of brush border membrane vesicles of the pig small intestine has been carried out in order to, subsequently, assess the intestinal digestibility of a series of commercial galactooligosaccharides differing in the predominant glycosidic linkage, as well as that of novel lactulose-derived galactooligosaccharides. In this sense, to a greater or lesser extent, all carbohydrates tested presented degradation rates by small intestinal enzymes.

Crucial structure-function relationship was found by determining the key role of structural features (glycosidic linkage or monosaccharides composition) on their resistance to degradation, increasing therefore essential knowledge towards the eventual development of new customized prebiotics. On the other hand, interest towards the production and obtainment of prebiotics using more sustainable methods has also raised a great interest. Therefore, in a second part of this dissertation, the prebiotic potential of pectic compounds obtained from agricultural by-products of artichoke, sunflower and citrus products was evaluated. A high prebiotic effect similar to well-recognized prebiotics was revealed after the *in vitro* fermentation study. Moreover, structure-function relationships were also found by elucidating the effect of the structural features (molecular weight, methylation degree, monosaccharide composition) on their ability to grow beneficial bacteria. Findings in this part of the thesis provided evidence on the prebiotic potential of theses substrates and may enhance the use of waste agricultural by-products as a renewable source of bioactive compounds.

Resumen

La modulación de la microbiota intestinal a través del uso de prebióticos ha sido ampliamente estudiada en las últimas décadas debido a su relación con la salud humana. Existen numerosos estudios acerca de los efectos beneficiosos de estos prebióticos sobre la microbiota intestinal, sin embargo, el conocimiento actual sobre su paso previo por el tracto gastrointestinal superior y, más concretamente, sobre los posibles cambios estructurales durante el proceso de digestión gastrointestinal es muy limitado, dando por hecho su total resistencia . En ese sentido, una primera parte de esta tesis doctoral está enfocada en el estudio de la digestibilidad de distintos carbohidratos prebióticos reconocidos (*Capítulos 1-3*). Se ha propuesto un método *in vitro* fiable y eficaz, basado en la utilización de extractos intestinales de rata, para evaluar la digestión de estos carbohidratos que solventaría importantes limitaciones que presentan los métodos actualmente en uso y que ignoran el papel clave de las carbohidrasas expresadas en la mucosa intestinal. Asimismo, y dado que el cerdo es un mamífero fisiológicamente más similar a la especie humana que la rata, se ha procedido al aislamiento de vesículas del epitelio intestinal de cerdos para, posteriormente, evaluar la digestibilidad intestinal de galactooligosacáridos comerciales caracterizados por diferentes enlaces glicosídicos, así como de nuevos galactooligosacáridos derivados de lactulosa. Los resultados de esta primera parte resaltan la idoneidad del empleo de estos extractos para la simulación de la digestibilidad intestinal de carbohidratos. Se ha observado una degradación, en mayor o menor medida, de los prebióticos analizados tras el tratamiento con las

enzimas intestinales y, además, se han establecido correlaciones importantes entre la estructura química de estos compuestos (composición monomérica, tamaño molecular o enlace glicosídico) y su resistencia a la degradación durante la digestión intestinal. Por otro lado, y debido al interés creciente hacia el desarrollo de nuevas estrategias sostenibles para la obtención de prebióticos emergentes, la segunda parte de esta tesis se ha centrado en la evaluación del potencial prebiótico de sustratos alternativos, como son los compuestos pécticos, obtenidos a partir de subproductos de la industria agrícola de alcachofa, girasol y cítricos. Para ello, se han utilizado modelos de fermentación *in vitro* estáticos y dinámicos que suponen un avance debido a la posibilidad de simular las diferentes etapas gastrointestinales en un entorno fisiológicamente más relevante. En general, se observó una alta actividad prebiótica en los sustratos analizados, siendo similar a la de prebióticos reconocidos. Además, ha sido posible correlacionar determinadas características estructurales (tamaño molecular, grado de metilación y composición de monosacáridos) con su capacidad para promover el crecimiento de bacterias beneficiosas. De este modo, los resultados obtenidos en esta parte aportan evidencias sobre el potencial prebiótico de estos sustratos y apuntan hacia la potencial utilización de determinados subproductos agrícolas como fuentes sostenibles para la obtención de compuestos bioactivos.

GENERAL INTRODUCTION

1. General Introduction

1.1 The Gastrointestinal Microbiota

Human body hosts over trillions of bacteria, viruses, fungi and other microorganisms, conforming together the microbiome that constitutes 90% of the total number of cells associated with our body and only the remaining 10% are human cells (Ley et al, 2006). These microorganisms represent a source of genetic diversity causing that two human microbiomes are not considered to be the same.

Among the different sites of the human body inhabited by these microorganisms such as, the mouth, throat and airways, skin or the urogenital system, the digestive gastrointestinal tract represents the most important colonized source of microbial stimulation of the human body (Grice, Segre 2012; Hooper et al, 2001). Over 10^{14} microorganisms colonize the gastrointestinal tract and continuously engage in a dynamic dialog with the host cells (Marchesi, Shanahan, 2007). In this sense, the total mucosal surface of an adult human gastrointestinal tract is up to 300 m^2, making it the largest body area interacting with these microorganisms, named “gut microbiota”. The metabolic activity performed by these microorganisms could be considered equal to a virtual organ, causing gut microbiota to be often referred as a “forgotten” organ (O’Hara, Shanahan, 2006). A fraction

estimated as < 30% of the gut microbiota was cultured in the last decades (Fraher et al. 2012). However, contemporary culture-independent techniques such as the introduction of microbial culturomics has increased our knowledge of the gut microbiota allowing to culture previous uncultured gut bacteria, providing an estimation of the microbial composition and biodiversity (Lagier et al. 2016; Lagier et al. 2015), although there is still a long way to investigate.

Figure 1 shows the highly dynamic behaviour and distribution of the gut microbiota. Different physicochemical characteristics of each anatomical region, such as pH, redox potential, availability of diet-derived compounds, transit rates or host secretions establishing the contrasting concentrations and prevalence of the bacteria in the gastrointestinal tract.

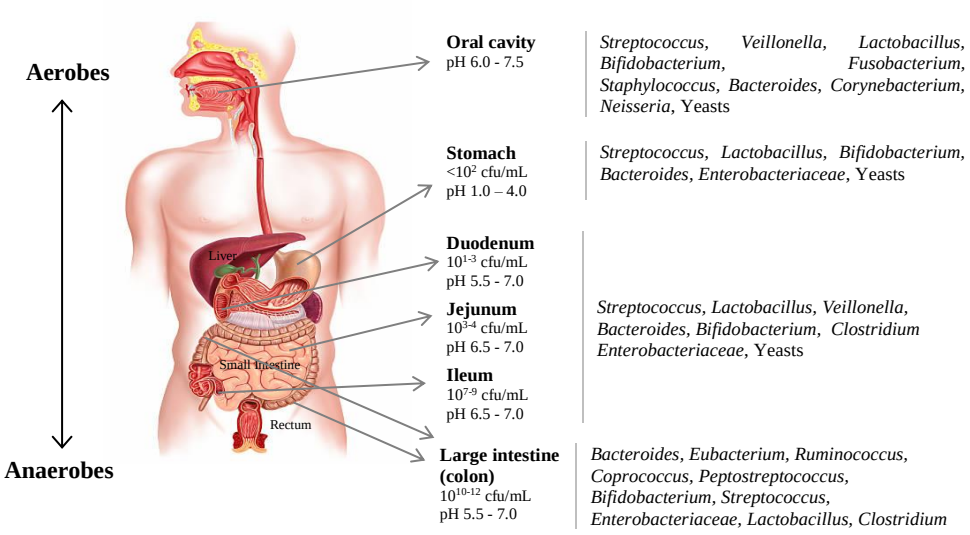

Figure 1. Key microbiological features (main genera and relative bacterial concentrations) and pH values of the human gastrointestinal tract. [cfu: colony-forming units]. Adapted from Iannitti and Palmieri (2010) and Kovatcheva (2013).

The upper gastrointestinal tract, consisting of the oral cavity, stomach, duodenum and jejunum, holds a scarce microbiota whose concentration is below 10^4 microorganisms (cfu) per mL of digesta. The relative low microbial population in this part is firstly affected by the fast flow of food in the mouth that causes a rapid wash out of the microbes. Moreover, the highly acidic conditions in the stomach and the subsequent release of bile acids and pancreatic enzymes in the duodenum limit the prevalence of these microorganisms. Microbial concentration increases toward the end of the small intestine and the colon reaching values of 10^7-10^8 and 10^{12} bacterial cells per mL, respectively. Slow transit time, higher exposure to nutrients at nearly neutral pH, and low redox potential provide a more favourable environment to bacterial colonization and growth in these gut regions (Donaldson et al 2016; Kovatcheva, 2013). However, microbial populations have been associated not only with the different anatomical regions and conditions of the gut but also with host genetics, age, diet, geographical location, medication intake, lifestyle and other environmental factors, as it is summarized in **Figure 2**.

The colonization of the human gut begins at birth. In this sense, initial colonization of the gut in infants appears to be dependent on delivery mode. Vaginally delivered babies acquire a microbiota similar to those of their mother's vagina (i.e., dominated by *Lactobaccillus* and *Prevotella*), and

babies delivered via caesarean section (C-section) acquire microbiota similar to those typically associated with the skin (i.e. *Propionibacterium*, *Staphylococcus*, and *Corynebacterium*) (Dominguez-Bello, et al 2010). Moreover, gestational age has been reported to impact on the infant gut, whereby a dominance of Proteobacteria, with high values of *Clostridium* and *Staphylococcus*, and much lower levels of Actinobacteria have characterized pre-term infants. In contrast, the full-term infant gut has been correlated with higher levels of *Bifidobacterium* and *Bacteroides*, which tend to be dominant in the early weeks of life (Arboleya et al, 2015; Barrett et al 2013).

Effects of breast feeding versus formula feeding have been also reported and babies that are solely breastfed until weaning tend to have a more stable, less diverse bacterial community with higher proportions of bifidobacteria than formula-fed babies (Klaassens et al, 2009). After the introduction of solid food, gut microbiota composition develops towards the adult pattern and resembles that of an adult by age 3 years with increased diversity and increased abundance of anaerobic Firmicutes (Fallani et al 2011). In this sense, early colonization of the gut has been shown to influence maturation of the immune system. Lastly, Bacteroidetes become more abundant and Firmicutes decrease in elderly adults (aged > 65 years) compared with younger adults (Claesson et al, 2011). In addition, a decline in microbiota diversity has been reported in old age, with reducing numbers of

bifidobacteria and increases in Enterobactericeae (Woodmansey, 2007) (**Figure 2)**.

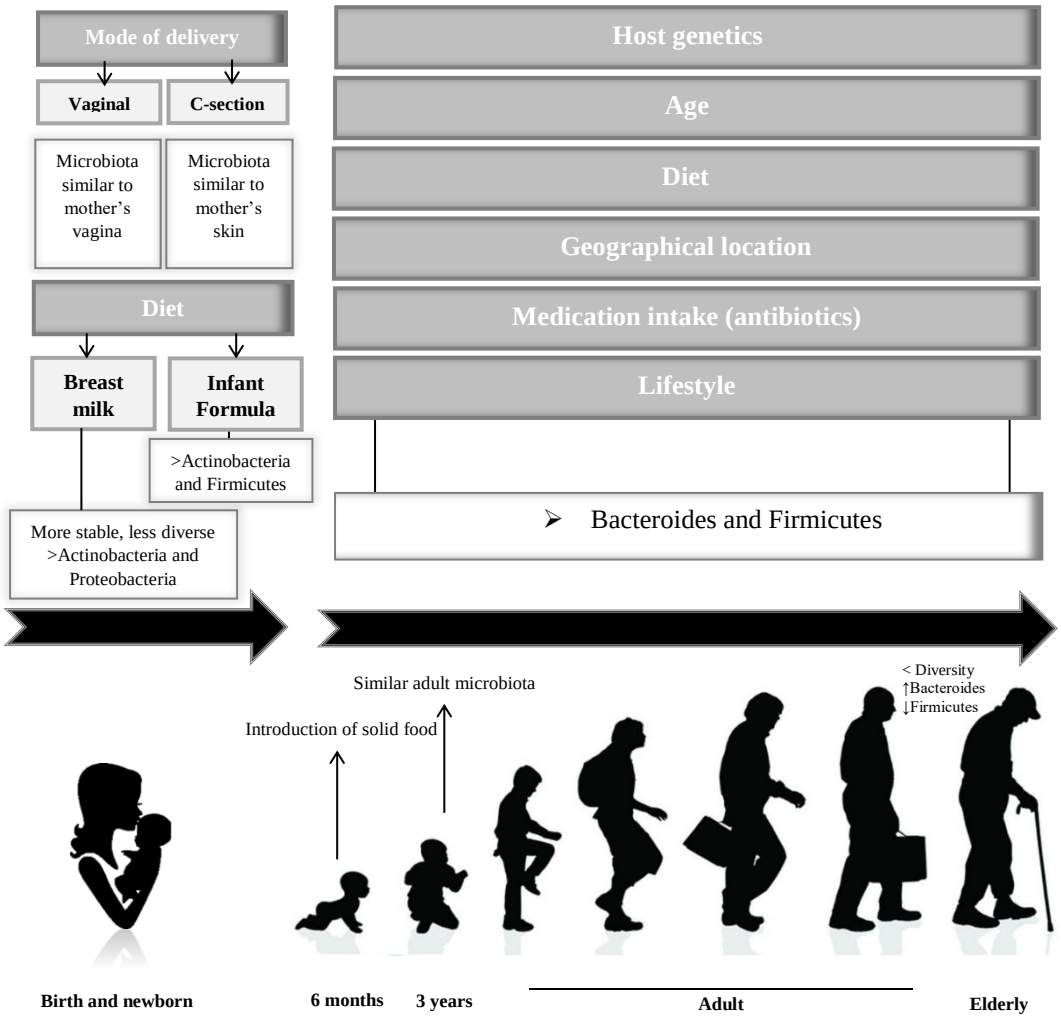

Figure 2. Variability of microbiota and factors involved in microbiota establishment. Adapted from Villanueva-Millán et al, 2015; Municio 2015.

1.1.1 Gut microbiota and health

As with any other ecological system, microorganisms in the gastrointestinal tract live in a natural balance called “symbiosis”. Bacteria such as “symbionts”, which have a mutualistic relationship with the host (that is, both are beneficial to each other); “commensal”, which do not provide benefit but neither harm the host, and some potentially pathogenic microorganism, coexist in perfect equilibrium. In this sense, microbiota of healthy individuals is known to confer a number of health benefits relating to, for example, pathogen protection, nutrition, host metabolism or immune modulation (**Figure 3)** (Blum, 2017). The perspective that the gut microbiota may have an important role in both human health and disease was by no means a new concept, as the Russian scientist Elie Metchnikoff already a century ago indicated the clinical importance of the host colonic microbiota, suggesting that certain microorganisms might promote health.

However, when the balance is altered causing changes in the bacterial composition, diversity or function (either a reduction in the numbers of symbiont and/or an increase in the numbers of pathogenic microorganisms), “dysbiosis” occurs. In this case, the gastrointestinal microbiota loses its protective capabilities and can lead to several diseases such as Crohn’s disease, colorectal cancer, irritable bowel syndrome, celiac disease, obesity,

diabetes or simply digestive discomfort such as bloating, flatulence, and abdominal pain (Wang et al, 2017).

In view of the numerous and diverse physiological functions of the intestinal microbiota in human health, it is not surprising that, in recent years, microbiota has also been related with other very important diseases such as bone diseases (Ibañez et al 2019), where the gut microbiota has been pointed out as a major regulator of the bone mineral density, and also several neurological/psychiatric diseases (Blum, 2017).

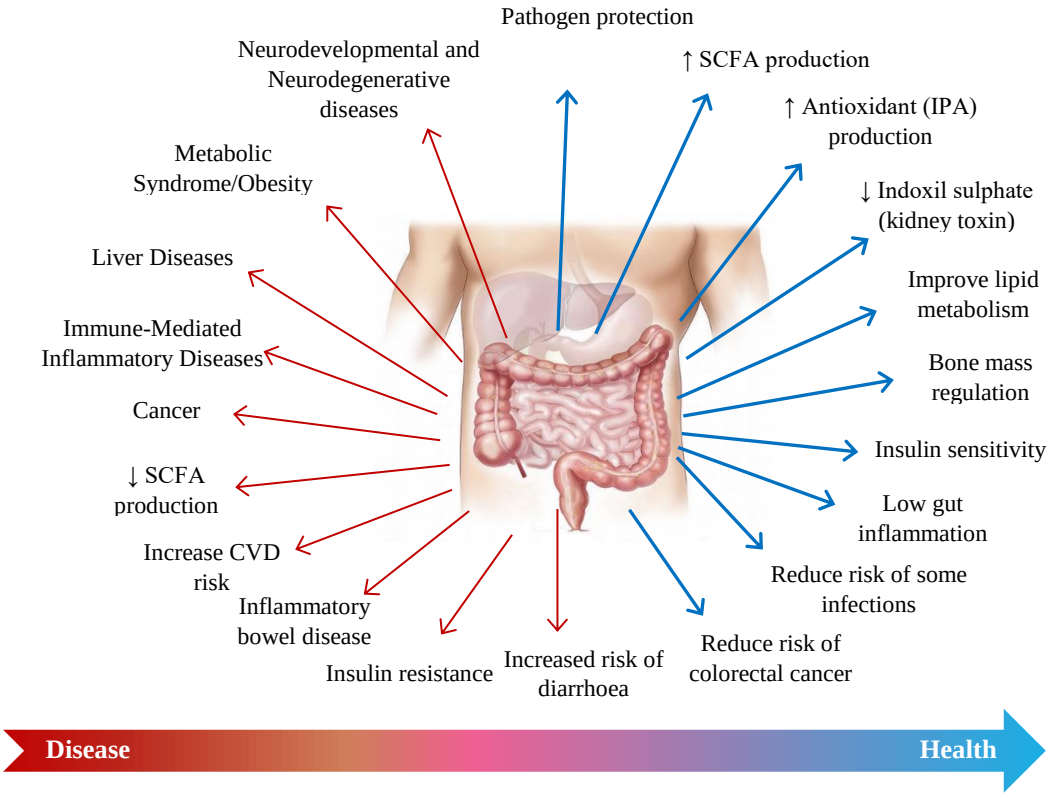

Figure 3. Schematic representation of the role of the gut microbiota in health and disease by given some examples of the positive and negative effects. (IPA = indolepropionic acid; SCFA = short chain fatty acid).

1.1.1.1 The gut-brain axis

The complex microbiota-host interactions are very dynamic, involving a variety of mechanisms that include immune, hormonal, and even neural pathways. In this sense, the connection between the microbiota and the brain has been of great interest in recent years.

Through a bi-directional communication network with the brain, called the "microbiota gut-brain axis", the commensal bacteria contribute to maintain homeostasis of the central nervous system (CNS) and influence our behaviour and mood. Interactions mechanisms are slowly being elucidated but these include production of microbial metabolites and immune mediators, such as cytokines and signalling directly to the brain via the vagus nerve (Dinan et al, 2015; El Aidy et al, 2014; Sherwin et al, 2016) (**Figure 4**).

The vagus nerve plays a crucial role in facilitating this bidirectional communication and it represents the main afferent pathway from the abdominal cavity to the brain. The vagus nerve transmits the information from the luminal environment to the CNS. In this sense, neurochemical and behavioural effects are not present in vagotomised mice, recognising the vagus nerve as a major modulatory constitutive communication pathway between microbiota and brain (Bravo et al, 2011). Moreover, bacterial commensal microbiota can also communicate with the brain via regulating

neurotransmission. Microbiota produces molecules that act as local neurotransmitters such as GABA (γ-Aminobutyric acid), which represents the main inhibitory neurotransmitter in the CNS, and dysfunctions in this transmitter system are linked to mood disorders, such as depression, anxiety and autism (Möhler, 2012). Microbiota can also synthesize histamine, which is linked to central processes such as circadian rhythms, food intake, learning and pain perception (Brown et al 2001). Microbiota could also contribute to the production of catecholamines, such as dopamine and noradrenaline (Asano et al 2012; Matsumoto et al 2013), whose dysfunction has been linked to various neurological and psyquiatric disorders, such as Parkinson's disease and depression. Lastly, bacterial metabolites (SCFAs) such as butyric, propionic and acetic acids have shown to be able to stimulate sympathetic nervous system (Kimura et al 2011). In fact, the brain-affecting disorders, where the intestinal microbiome and enteric nervous networks are actively involved are quite numerous (**Table 1)**.

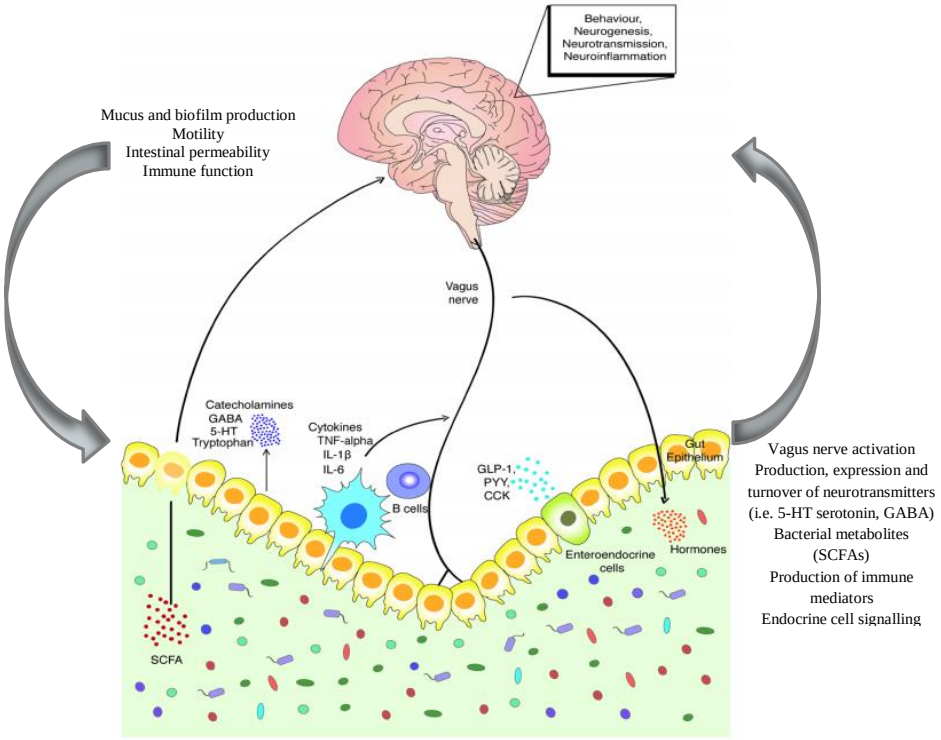

Figure 4. Key communication pathways of the microbiota gut-brain axis. Putative mechanisms by which bacteria access the brain and influence behaviour include bacterial products that gain access to the brain via the bloodstream and the area postrema, via cytokine release from mucosal immune cells, via the release of gut hormones such as 5-hydroxytryptamine (5-HT) from enteroendocrine cells, or via afferent neural pathways, including the vagus nerve. On the other hand, stress and emotions can influence the microbial composition of the gut through the release of stress hormones or sympathetic neurotransmitters that influence gut physiology and alter the normal habitat of the microbiota. *GABA (γ-Aminobutyric acid), TNF tumour necrosis factor, IL interleukin, GLP glucagon-like peptide, PYY peptide YY, CCK cholecystokinin.* Figure adapted from Sherwin et al (2016) and reproduced with kind permission from Springer.

The main mechanisms for the brain to influence the gut are mediated by the alteration of the normal luminal/mucosal habitat. In this sense, the brain has a prominent role in the modulation of gut functions, such as motility, secretion of acids, bicarbonates and mucus and mucosal immune response (Macfarlane, Dillion, 2007); thus, a dysregulation on the gut-brain axis can affect gut microbiota through the perturbation of the normal mucosal

habitat. In addition, brain might also affect microbiota composition and function by alteration of the intestinal permeability, by allowing bacterial antigens to pass through the epitelium and stimulate an immune response in the mucosa (Demaude et al 2006).

Table 1. Summary of brain-affecting disorders, where gastrointestinal manifestation exists and the intestinal microbiome and enteric nervous are actively involved. Table modified from Lerner et al. (2017)

Parkinson's disease	[Sun et al. (2018); Perez-Pardo et al (2017); Cersosimo et al, (2013); Mulak et al, (2015); Poirier et al, (2016); Rietdijk et al, (2017); Houser et al, (2017)]
Autism spectrum disorder	[Li et al, (2017); Luna et al, (2016); Li et al, (2016); Kraneveld et al, (2016)]
Amyotrophic lateral sclerosis	[Wright et al. (2018); Fang et al. (2016); Wu et al. (2015); Luesma et al. (2014)]
Alzheimer diseases	[Mancuso et al. (2018); Vogt et al. (2017); Jiang et al. (2017); Shoemark et al. (2015); Friedland (2015)]
Prion diseases	[Bradford et al. (2017); Davies et al. (2006); Albanese et al. (2008); Donaldson et al. (2016); Natale et al. (2011)]
Creutzfeldt-Jakob disease	[Donaldson et al. (2016); Beekes et al. (2007)]
Transmissible spongiform encephalopathies	[Friedland (2015); Donaldson et al. (2016); Beekes et al. (2007); Natale et al. (2011)]
Additional conditions that affect the microbiota	
Depression	[Clapp et al. (2017); Jiang et al. (2015); Zheng et al. (2016); Foster et al. (2013); Luna et al. (2015); Dinan et al. (2013)]
Anxiety	[Clapp et al. (2017); Foster et al. (2013); Luna et al. (2015); Bercik et al. (2011)]
Behaviour	[Dinan et al. (2015); Zeng et al. (2016); Cryan et al. (2011)]
Cognition	[Proctor et al. (2017); Esposito et al. (2016); MacFabe et al. (2011)]
Mood	[Dinan et al. (2017); Dinan et al. (2016); MacQueen et al. (2017)]
Stress	[Luna et al. (2015); Bauer et al. (2016); Moloney et al. (2014); Fujikawa et al. (2015)]
Fatigue	[Nagy-Szakal et al. (2017); Giloteaux et al. (2016); Van Erp et al. (2017); Lakhan et al. (2010)]
Aging	[Buford (2017); Shoemark et al. (2015)]

Thereby, regulation or restoring the gut microbiota is considered as a therapeutic tool for several of the diseases noted above. Nowadays, the two main methods of modifying the gastrointestinal microbiota include dietary compounds supplementation (prebiotics) and supplementation of live microorganisms, which have shown to positively influence health (probiotics). Moreover, the combination of these two methods to obtain synergistic benefits has been named “synbiotics”.

1.1.2 Probiotics

Élie Metchnikoff (a Russian scientist) and Henry Tisser (a French paediatrician) were the first to make suggestions concerning a positive role played by some bacteria during the early years of the 1900s. However, it was not until 1965 when Lilly & Stillwell first introduced the term “probiotic” that in contrast to antibiotics, defined probiotics as “microbially derived factors that stimulate the growth of other organisms”. The word “probiotic”, which is translated from the Greek, meaning “for life”, was redefined by Parker (1974) as “organisms and substances which contribute to intestinal microbial balance”, later on by Fuller (1989) as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. This last definition removed the reference to

particles and a probiotic would therefore incorporate living microorganisms, seen as beneficial for gut health, into the diet.

Nowadays, the most widely adopted definition of probiotics was proposed in 2001 at an Expert Consultation meeting arranged by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) (FAO/WHO). This definition was ratified by the International Scientific Association for Probiotics and Prebiotics consensus (ISAPP) in a report in 2014 (Hill et al. 2014) with a minor grammatical correction as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host".

The most common types of microorganisms used as components of probiotics preparations are lactic acid bacteria, such as *Lactobacillus* and *Bifidobacterium*, although other bacteria and certain yeasts are also used (Didari et al. 2014). Most of these health-promoting bacterial strains are normal residents or common transients of the human digestive system and as such do not display infectivity or toxicity. The most common probiotic microorganisms with claimed health benefits for humans are noted in **Table 2**.

Table 2. Relevant strains of probiotic microorganisms. Table adapted from Fijan (2014).

Genus	Species
Lactobacillus	*L. rhamnosus, L. acidophilus, L. plantarum, L. casei, L. delbrueckii* subsp. *bulgaricus, L. brevis, L. johnsonii, L. fermentum, L. reuteri*
Bifidobacterium	*B. infantis, B. animalis* subsp. *lactis, B. bifidum, B. longum, B. breve*
Others	*Saccharomyces boulardi, Lactococcus lactis* subsp. *lactis, Enterococcus durans, E. faecium, Streptococcus thermophilus, Pediococcus acidilactici, Leuconostoc mesenteroides, Bacillus coagulans, B. subtilis, B. cereus, Escherichia coli* Nissle 1917.
Emerging genera	*Faecalibacterium prausnitzii, Eubacterium rectale, Roseburia spp., etc.*

However, as probiotics involve live colonic microorganisms, to be a good candidate these should fulfil certain requirements. Microorganisms must retain their properties during large-scale industrial preparation, it should also remain viable and stable during storage and use, be recognized as "generally regarded as safe" (GRAS) and naturally the organisms should be able to survive in the gastrointestinal conditions such as the low gastric pH and the digestive enzymes and bile salts activity.

There are several reports regarding the positive effects associated with the intake of probiotics; however, probiotics may frequently present different performances between strains, where some of them have shown a limited colonization of the intestine or even a limited resistance to gastrointestinal conditions. According to this, species such as *Lactobacillus*, for example, are

broadly resistant, whereas some strains of *Bifidobacteria* are extremely sensitive to low pH, exhibiting low or no survival rates at pH 2 and pH 3 (Fontana et al. 2013; Sanz 2007; Takahashi et al. 2004). For these reasons, a very good alternative approach for microbiota management through diet is the use of "prebiotics" which are directed toward specifically changes in the gut microbiota composition.

1.2 Prebiotics

The prebiotic concept was initiated to uphold the probiotic concept. The prebiotic concept may also overcome the major drawback of probiotics, which is to ensure a high viability in the product and, therefore, to have a robust survival rates in the gut. In this way, the target bacteria are already in the host and prebiotics stimulate its growth.

Back to 1921, almost 100 years ago, Rettger & Cheplin described assays with humans whose microbiota was enriched with lactobacilli following consumption of carbohydrates. However, it was not until 1995 when the prebiotic concept was defined for the first time and, later on, it has been refined and validated several times. Gibson & Roberfroid (1995) defined a prebiotic as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon". In this sense,

whereas probiotics use live microorganisms, prebiotics were defined as non-viable substrates that serve as nutrients for beneficial microorganisms harboured by the host. **Table 3** shows the evolution and the most relevant changes to keep the concept updated and to expand the original idea of the prebiotic definition over time.

Table 3. Evolution and changes of the definition of prebiotic over time.

1995 - "Non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon". *Gibson G.R. & Roberfroid M.B. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. **1995,** J. Nutr. 125: 1401-1412.*
2004 - "Selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" *Gibson G.R., Probert H.M., Van Loo J.A.E., Rastall R.A. & Roberfroid M.B. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. **2004,** Nutr. Res. Rev. 17: 259-275.*
2008 - "A non-viable food component that confers a health benefit on the host associated with modulation of the microbiota" *Pineiro, M., Asp N.G., Reid G., Macfarlane S., Morelli L., Brunser O. & Tuohy K. Food and Agriculture Organization of the United Nations (FAO) technical meeting on prebiotics. **2008**, J. Clin. Gastroenterol. 42, S156–S159.*
2010 - "A selectively fermented ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" *Gibson G.R., Scott K.P., Rastall R.A., Tuohy K.M., Hotchkiss A., Dubert-Ferrandon A., Gareau M., Murphy E.F., Saulnier D., Loh G., Macfarlane S., Delzenne N., Ringel Y., Kozianowski G., Dickmann R., Lenoir-Wijnkoop I., Walker C., Buddington R. Dietary prebiotics: current status and new definition. **2010**, Food Sci. Technol. Bull.- Funct. Foods, 7: 1-19*
2015 - "A non-digestible compound that, through its metabolization by microorganisms in the gut, modulates the composition and/or activity of the gut microbiota, thus, conferring a beneficial physiological effect on the host" *Bindels, L.B., Delzenne, N M., Cani, P.D. & Walter, J. Towards a more comprehensive concept for prebiotics**. 2015**. Nat. Rev. Gastroenterol. Hepatol. 12, 303–310*
2017 - "A substrate that is selectively utilized by host microorganisms conferring a health benefit" *Gibson G.R., Hutkins R., Sanders M.E., Prescott S.L., Reimer R.A., Salminen S.J., Scott K., Stanton C., Swanson K.S., Cani P.D., Verbeke K. & Reid G. The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. **(2017**). Nat. Rev. Gastroenterol. Hepatol. 14, 491-502.*

Therefore, the most recently proposed definition by the panel of experts of the ISAPP in 2017 remarks prebiotics as “a substrate that is selectively utilized by host microorganisms conferring a health benefit”. In this sense, this new definition includes prebiotics to interact with extraintestinal microbiota, such as the located in the vagina or skin. Even more, this new definition opens the field for candidates structurally different from carbohydrates. In consequence, in addition to carbohydrate-based compounds that have been traditionally considered as top prebiotics, other substances such as polyphenols and polyunsaturated fatty acids might fit the updated definition assuming convincing weight of evidence in the target host.

1.2.1 Prebiotic substrates

By definition, the prebiotic concept emphasises the specific stimulation of host microorganisms leading to a health benefit. In this sense, the criterion of selective utilization differentiates prebiotics from many other substrates that can also affect the microbiota (Roberfroid et al. 2010). Selectivity does not necessarily mean effects on just one microbial group; a selective effect could extend to several microbial groups. As based on the evidence so far, prebiotic targets extend beyond stimulation of bifidobacteria and lactobacilli, and recognizes that health benefits can derive from

stimulating other beneficial bacteria such as *Roseburia*, *Eubacterium* or *Faecalibacterium* spp. for example (Gibson et al. 2017).

Thus, to be considered a prebiotic, the involved compound must fulfil the following criteria, which should be adequately evidenced by *in vitro* and *in vivo* studies: a) A prebiotic must be selectively utilized by host microorganisms, b) A prebiotic must have an adequate evidence of its health benefits on the target host and, c) Dietary prebiotics *should be non-digested* by the host but utilized by the microbiota.

To date, the main substrates for bacterial modification are dietary non-digestible carbohydrates that evade upper gastrointestinal hydrolysis and absorption. It is noteworthy to mention that resistance to degradation of these substrates contributes positively to the regulation of the caloric intake and diminishes the absorption of free monosaccharide that are usually released and absorbed in the small intestine. These non-digestible carbohydrates include dietary fibres such as resistant starch/dextrin, non-starch polysaccharides (i.e. pectins, arabinogalactans, hemicellulose), non-digestible oligosaccharides of plant origin (inulin and oligofructoses), as well as undigested disaccharides (i.e. lactulose) and sugar alcohols (i.e. lactitol and isomalt). However, the most extensively prebiotics documented to have health benefits in humans and, consequently, the most widely accepted are

lactulose, inulin, and non-digestible oligosaccharides (NDOs): fructooligosaccharides (FOS), galactooligosaccharides (GOS), and the human milk oligosaccharides (HMOs)

1.2.1.1 Dietary fibre

According to the Codex Alimentarius Commission, a dietary fibre is defined in the European Union as: "carbohydrate polymers with three of more monomeric units, which are neither digested not absorbed in the human small intestine and belong to the following categories: a) edible carbohydrate polymers naturally occurring in foods as consumed, b) edible carbohydrate polymers which have been obtained from food raw materials by physical, enzymatic, or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence, and c) edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence" (Codex Alimentarius, 2010). In this sense, dietary fibre refers to a highly chemical heterogeneous group of substances that are resilient to upper gastrointestinal digestion and absorption. However, categorizing fibres as prebiotics would be inappropriate due to their higher variability and heterogeneous behaviour.

Nevertheless, certain soluble fibres have shown to have a prebiotic effect being considered as prebiotics (inulin-type fructans and GOS) and

some others are being considered as prebiotic candidates (polydextrose, pectins, pectic-oligosaccharides, polysaccharides from algae) (Míguez et al 2016).

1.2.1.1.1 Inulin

Inulin is naturally present in several foods such as chicory, Jerusalem artichoke, garlic, artichoke, onion, wheat, banana, and oats as well as soybean. Inulin-type fructans are oligo-/polymers of D-fructose joined by β(2→1) bonds with an α(1→2) linked D-glucose at the terminal end of the molecule. Molecules with DP between 3 and 10 are referred to as oligofructoses, and those with a DP between 10 and 65 are known as inulin.

The prebiotic activity of inulin-type fructans has been widely confirmed (Kolida & Gibson 2007). This is mainly because these compounds are preferably metabolized by bifidobacteria, which are able to break down and utilize these substrates due to their β-fructosidase enzymes. In this context, chicory inulin has received an EU health claim: “Inulin improves bowel function” endorsed by the EFSA (EFSA, 2015).

1.2.1.1.2 Fructooligosaccharides (FOS)

Concerning non-digestible oligosaccharides (NDOs), FOS represent the oligomers formed of D-fructose joined by β(2→1) bonds with an α(1→2) linked D-glucose at the terminal end of the molecule. These oligosaccharides

are obtained by degradation of chicory inulin or using sucrose as substrate in a transfructosylation process. In the first approach, chicory inulin is cleaved randomly by microbial endoinulinases (EC 3.2.1.7) yielding oligofructosides (F_n series) with a DP between 2 and 10. In the second approach, cane sugar or beet sugar is fructosylated to GF_2, GF_3, and GF_4 by β-fructofuranosidase (EC 3.2.1.26) or β-fructosyltransferase (EC 2.4.1.100) from microbial origin (Ganaie et al. 2014; Bali et al. 2015; Flores-Maltos et al. 2016). Similar to inulin, it is generally accepted that FOS reach the colon where it can selectively stimulate the beneficial host microorganisms, especially bifidobacteria (Kolida & Gibson 2007).

1.2.1.2 Galactooligosaccharides (GOS)

GOS are prebiotic compounds that are currently produced by the enzymatic transgalactosylation of lactose with β-galactosidase (E.C. 3.2.1.23) from different microbial strains. Transgalactosylation process in GOS is similar to the obtainment of FOS using sucrose as substrate. In this process, the enzyme β-galactosidase hydrolyses lactose and, instead of transferring the galactose unit to the hydroxyl group of water (hydrolysis), the enzyme transfers galactose to another carbohydrate, in this case lactose, to result in oligosaccharides with a higher degree of polymerization (DP) (Kim et al. 1997) (**Figure 4**).

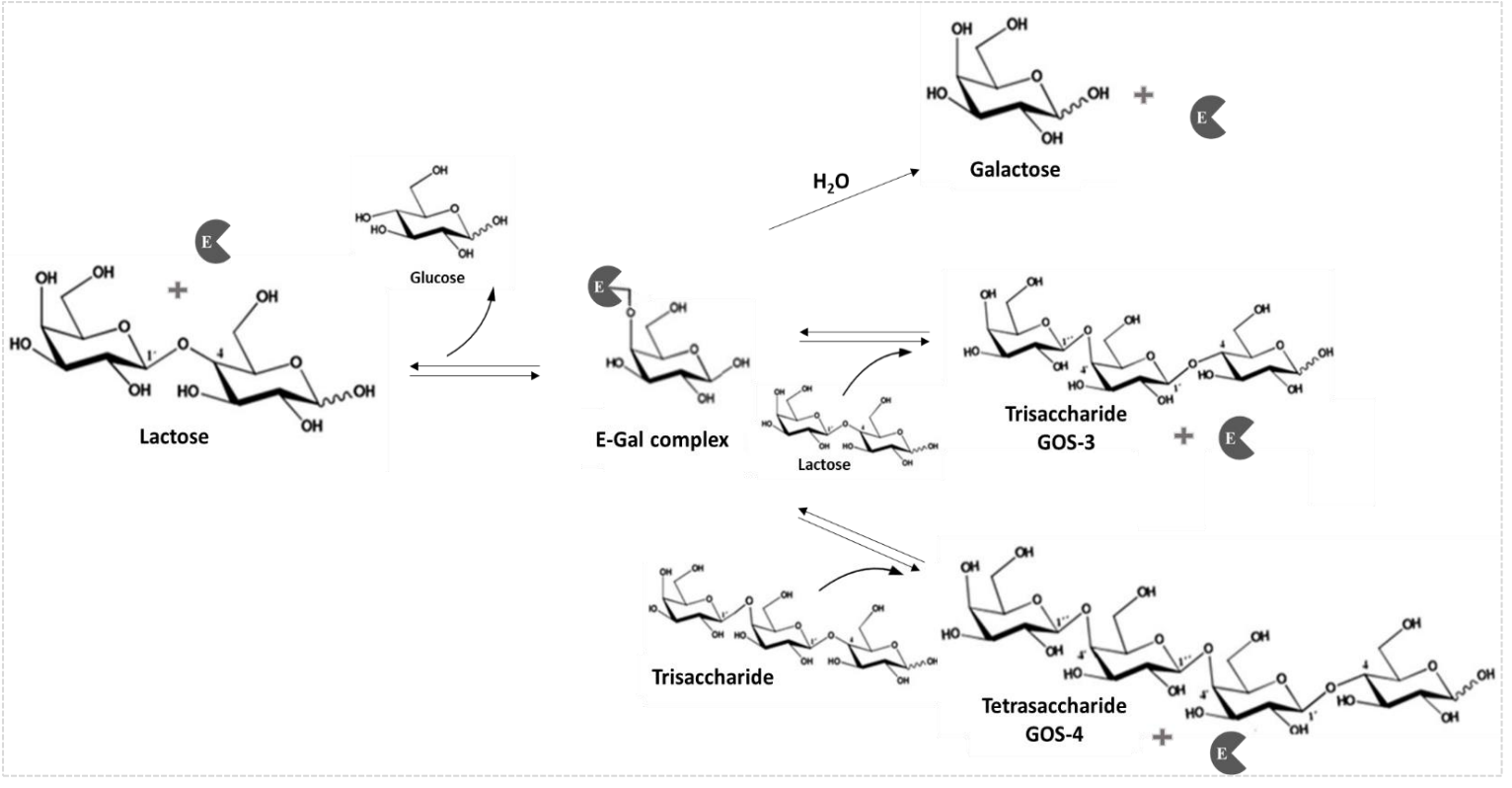

Figure 4. Schematic representation of the reaction mechanism of GOS synthesis by transgalactosylation with β-galactosidases (E). Adapted from Vera et al. (2016).

Regarding the synthesis reaction, lactose hydrolysis and transgalactosylation are concomitant reactions catalysed by β-glycosidase, therefore resulting products of the reaction will be monomeric products, as well as many newly formed β-glycosides, mainly di-, tri-, and tetrasaccharides (Prenosil et al. 1987). In this regard, it is important to notice the high caloric value and glycaemic index of these monosaccharides that sometimes are removed. GOS obtained after reaction are constituted by a complex mixture of galactoses linked by different linkages β(1↔1), β(1→2), β(1→3), β(1→4) and β(1→6) and can vary from 1 to 8 units and a terminal glucose (Moreno et al. 2014). In this sense, composition of GOS mixture have shown to be deeply affected by several factors, such as the enzyme source, lactose concentration, substrate composition and reaction conditions (temperature, time and pH) (Moreno et al. 2014; Gänzle 2012; Torres et al. 2010). These compounds present a widely recognised prebiotic effect since they selectively stimulate the growth of lactic acid bacteria and bifidobacteria in the gut (Roberfroid et al. 2010). Moreover, GOS has been recognised as dietary ingredients and not as additives due to the presence of galactose-based oligosaccharides, which are also present in human milk oligosaccharides (HMOs).

1.2.1.3 Human Milk Oligosaccharides (HMOs)

HMOs are key constituent of human milk. They represent a structurally and biologically diverse group of complex indigestible sugars (Bode et al. 2006). HMOs are composed of both neutral and anionic species with building blocks of 5 monosaccharides: D-glucose, D-galactose, *N*-acetylglucosamine, *L*-fucose and *N*-acetylneuraminic acid. The basic structure as well as in GOS, includes lactose core at the reducing end and are elongated by *N*-acetyllactosamine units, with greater structural diversity provided by extensive fucosylation (Newburg et al. 2005). More than 200 different oligosaccharides have been identified to date, varying in size from 3 to 22 monosaccharide units (Bode 2012; Bode 2015). These compounds reach the colon where they are preferred substrates for several species of gut bacteria producing SCFA and promoting the growth of beneficial bacteria. HMOs has also shown to directly modulate host-epithelial responses, favouring reduced binding of pathogenic microbiota to the gut epithelium (Smilowitz et al 2014). Moreover, HMOs has been attributed as the main responsible of the higher presence of Actinobacteria, mainly represented by *Bifidobacterium* genus in breastfed infants, compared to formula-fed infants (Filippo et al 2010).

1.2.1.4 Lactulose and oligosaccharides derived from lactulose

Lactulose, 4-β-D-galactopyranosyl-D-fructose, represents the simplest recognised prebiotic. Lactulose does not occur naturally and it is obtained by isomerization of lactose where the glucose moiety is transformed to fructose in alkaline conditions (Zokaee et al. 2002) or by enzymatic methods, using β-glycosidases in a bioconversion process or β-galactosidases in a transgalactosylation mechanism in presence of fructose (Wang et al. 2013). Moreover, regarding the latter method using β-galactosidases from certain microorganism, such as *Aspergillus oryzae*, new potential prebiotic oligosaccharides can be synthesized by a direct reaction of transgalactosylation of lactulose (OsLu) in a similar way to GOS from lactose (Martinez-Villaluenga et al. 2008; Hernandez-Hernandez et al. 2011). As in the case of transgalactosylation of lactose, the released galactose moiety from lactulose is linked by β(1→6) and β(1→3), β(1→1) or β(1→4) glycosidic bonds to the galactosyl or fructosyl moiety of another lactulose molecule (**Figure 5**).

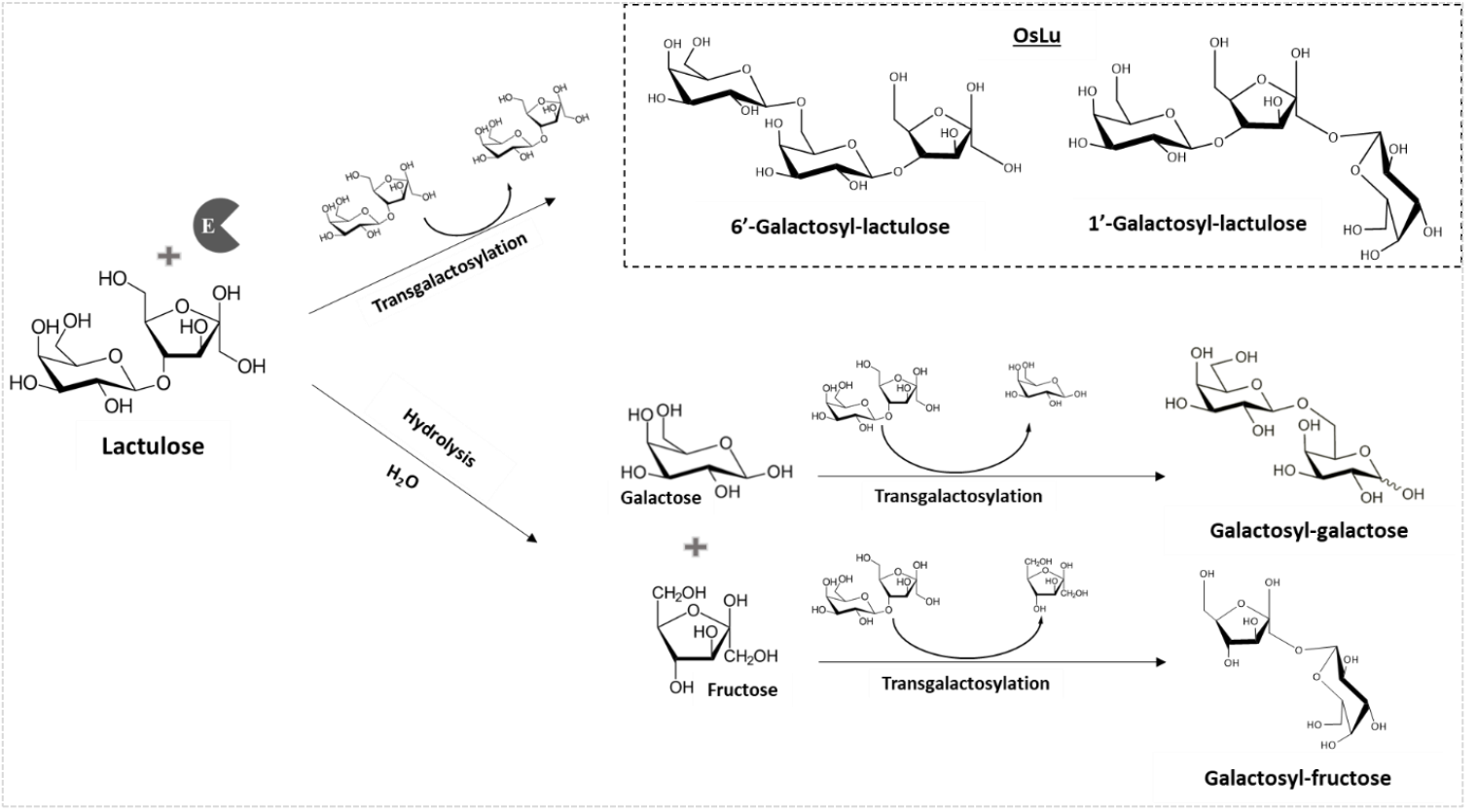

Figure 5. Schematic representation of the reaction mechanism of oligosaccharides from lactulose synthesis by transgalactosylation with β-galactosidases (E). Adapted from Diez-Municio et al. (2014).

Therefore, oligosaccharides derived from lactulose (OsLu) comprises a mixture of oligosaccharides with different linkages (mainly β(1→6)) containing one fructose residue (Martínez-Villaluenga et al. 2008; Hernádez-Hernández et al. 2011; Padilla et al. 2012; Cardelle-Cobas et al. 2016; Yin et al. 2018).

As the rest of prebiotics, lactulose presents a high resistance to the upper gastrointestinal degradation reaching the colon where it selectively stimulates the growth of beneficial bacteria and decreases in the counts of clostridia, streptococcus and enterobacteria (Gibson et al. 2000). It is well known that the resistance of oligosaccharides towards microbial degradation depends on their glycosidic linkages, monosaccharide composition, and degree of polymerization. Rapidly fermented carbohydrates mainly show a prebiotic effect in the caecum and proximal colon whereas the more slowly fermented oligosaccharides, such as OsLu, reach the distal colon and influence the microbial composition present there (Cardelle-Cobas et al. 2009a). In this sense, β(1→6) OsLu have shown to exert a better bifidogenic and anti-inflammatory effect than lactulose and GOS (Hernández-Hernández et al. 2012; Algieri et al. 2014; Li et al. 2015).

1.2.2 Biological properties and application of recognised prebiotics:

As previously stated in **section 1.1**, it is well established that the composition and activity of the microbiota significantly contribute to the health and well-being of the host. In this sense, prebiotics alter positively the composition of the intestinal microbiota, generally increasing the growth of beneficial bacteria such as bifidobacteria and lactobacilli. To date, there are a large number of publications reporting the possitive effect of recognised prebiotics on health and it continues increasing. Prebiotics' benefits to host health is managed by two principal mechanisms; the first one is related to the stimulation of the growth of beneficial bacteria in the gut and the other one is related to the production of short chain fatty acids (SCFA) (acetate, propionate, butyrate) during fermentation of carbohydrates (**see section 1.2.4**). The positive effects of prebiotic oligosaccharides have been largely reviewed in the literature and have been identified to play a key role in minimizing health-related risks, such as diabetes, cardiovascular disorders, cancer, acute infection, inflammation, gastrointestinal diseases and obesity (Cho et al. 2010; Slavin et al. 2013). Prebiotic consumption also enhances bioavailability of nutritionally important minerals such as calcium, magnesium, and iron (Slavin et al. 2013).

Regarding the application of recognised prebiotics, all prebiotic uses are situated as food ingredients. In this sense, the commercial market at present is dominated by inulin, FOS and GOS. FOS was approved as food ingredient in Japan since 1980 and has an approved FOSHU (Foods for Specified Health Uses) status. In the European Union has been recognised as food ingredient since 1991. FOS was first made commercially available in 1988 in the United States, it is considered GRAS (Generally Recognised as Safe) and it currently can be found in several food products worldwide under several trade names like Neosugar, NutraFlora®, Meioligo®, and Actilight, for example (Bali et al. 2015). Similarly, the use of GOS is increasing gradually in various applications worldwide. The first product for a specific target group was introduced in the early 1990s, when an infant formula containing GOS was launched (Cho et al. 2010). Since then, GOS are increasingly applied as ingredients for infant formula to mimic the biological functions of human milk oligosaccharides. Furthermore, because of their high solubility and stability (e.g., under pasteurization and sterilization conditions and in acid environments), GOS are particularly suitable for use in acid products, such as fruit juices, yogurts and heat-treated products such as bakery products. In general, prebiotics have been included in a wide variety of foods and supplements globally including beverages, dairy products

(yogurts, ice cream), desserts, bakery products, breakfast cereal, infant/toddler foods, food supplements (Cho et al. 2010).

1.2.3 Emerging and potential prebiotic compounds

At this stage, any dietary component that reaches the colon intact could be a potential candidate for prebiotic attribute; however, the criterion of selective utilization by the microbiota is the most difficult to fulfil. In this sense, there is a growing list of potential prebiotic compounds, and some of them are already commercialized such as, α-glucooligosaccharides, isomaltooligosaccharides (IMOS), lactosucrose, soy oligosaccharides and xylooligosaccharides (XOS) (Gibson et al. 2010). There is increasing evidence on the properties of these compounds even though more studies (i.e. human volunteer trials) are still needed to recognise these as prebiotics.

The growing interest towards prebiotic compounds and their effects to the host led to the pursuit of new compounds, which can be produced, through more efficient, sustainable, simple and less expensive processes for their application on a large scale. In this regard, other compounds with potential prebiotic effect, such as pectin and pectic-oligosaccharides, along with others like polydextrose (PDX), bacterial exopolysaccharides (EPS), polysaccharides from algae and sugar alcohols, are still in early stages of investigation.

1.2.3.1 Pectin

Pectins are a family of plant cell wall structural complex polysaccharides. Exact chemical composition of these compounds is still under debate, due to the high complexity of this molecule; however, their structure present common features, such as homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II). Pectins mainly consist of a galacturonic acid (GalA) rich backbone, known as homogalacturonan (HG ≈ 65%), which is partially methyl esterified in the carboxyl group at the C-6 position and O-acetyl-esterified in positions C-2 and C-3 (Mohnen, 2008). According to the amount of carboxyl groups that can be esterified with methyl groups, pectins are classified on the basis of their degree of esterification (DE) (Oakenfull 1991), also known as degree of methoxyl esterification (DM). Pectins with more than 50% of the carboxyl groups esterified are named as high methoxyl (HM) and pectins with less than 50% of carboxyl groups esterified are classified as low methoxyl (LM) (Thakur et al. 1997). Rhamnose (Rha) residues interrupt the HG structure to form rhamnogalacturonan-I (RG-I ≈ 20–35%) which is based on a backbone consisting of a repeating disaccharide of [→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→] residues. In addition, some rhamnose residues may contain sidechains that can vary from single glycosyl to polymeric of different types (1→5)-α-L-arabinans, (1→4)-β-D-galactans and arabinogalactans (Buffetto et al., 2015).

The highly branched nature of RG-I has led to the name of “hairy region”. RG-II consists of a linear backbone chain of galacturonic acid units, substituted with L-rhamnose, D-galactose and many unusual sugars, such as apiose, aceric acid, 3-O-methyl-L-fucose, 2-O-methyl-D-xylose, 3-C-carboxy-5-deoxy-L-xylose, 3-deoxy-D-manno-octulosonic acid and 3-deoxy-D-lyxo-heptulosaric acid (Palin et al. 2012; Yapo 2011). RG-II structure is the most structurally complex pectin domain and it is largely conserved across many plant species.

Most pectins are commercially obtained from citrus fruits like orange, lemons, grapefruit, and apples. These substrates contain high amounts of pectic substances. However, in recent years, the search of new sources of pectin appears very promising. In this sense, the use of waste by-products from the agricultural industry has become an interesting alternative for extraction of pectin, some examples include sunflower head residues, artichoke residues, and sugar beet pulp, among others (Muñoz-Almagro et al. 2018; Sabater et al. 2018; Ishii 1994).

Currently, pectins with no less than 65% GalA (FAO) are mainly used in the food industry as food additive (E440). However, the suitability of pectins for specific applications is governed by the structural features, including molecular weight (Mw), neutral sugar content, proportion of

HG:RG-I regions or the DM as these factors can affect their applicability as thickeners or as gelling and stabilizing agents (Gullón et al. 2013). Biological properties of pectins, and other dietary fibres, are mainly due to their non-digestibility in the gastrointestinal tract, reaching the hindgut where they are fermented by the colonic microbiota. In this sense, a positive effect of these compounds on gut beneficial microbiota has been observed after citrus and apple pectin fermentation, consisting of a growth of bifidobacteria and lactobacillus and an increase in SCFA (Jakobsdottir et al. 2013; Nazzaro et al. 2012; Olano-Martín et al. 2002; Dongowski et al. 2000).

1.2.3.1 Modified pectins and pectic-oligosaccharides (POS)

Pectin that has been treated with pH (low or high), heat or enzymes is generally referred to as 'modified pectin' (MP), although this term remains ambiguous, as pectin is a highly heterogeneous material and these modified pectin structures can vary widely depending on the pectin source, extraction and method modification. In this sense, it is generally understood that modifying pectin with heat, pH, enzymes or ultrasound (Díaz et al. 2007; Muñoz-Almagro et al. 2017; Muñoz-Almagro et al. 2018; Sabater et al. 2018) can decrease the molecular weight and proportionally increase the content in total neutral sugars.

Regarding biological properties, most of the studies addressing the bioactive effects of pectins have been carried out using a modified form of pectin. There are several investigations that have demonstrated a better bifidogenic effect and production of SCFA of lower molecular weight pectic compounds (Ho et al. 2017; Daguet et al. 2016; Khodaei et al. 2016), although neutral sugar content (arabinans and galactans) seems to play also a key role in their prebiotic potential (Di et al. 2017; Onumpai et al. 2001). Furthermore, there is growing evidence linking modified forms of pectic polysaccharides with anti-cancer activity, where pectins with a high neutral sugar content are more bioactive due to the hypothesis that galactan side-chains on pectin can bind to and inhibit the pro-metastatic protein galectin-3, resulting in the suppression of cancer cell proliferation, aggregation, adhesion and metastasis (Maxwell et al. 2012; Maxwell et al. 2016; Park et al. 2017; Nangia-Makker et al. 2002). Other beneficial health properties might include the reduction of atherosclerotic lesions (Lu et al., 2017), anti-inflammatory and antioxidant properties (Popov & Ovodov, 2013; Ramachandran, et. al. 2017) or immunostimulatory properties (Vogt et al., 2016). Taken that into account, to date there are two forms of modified citrus pectin commercialized obtained by chemical and enzymatic methods, GCS-100 (~ 10 kDa) patented as a mammalian anticancer agent (Raz & Pienta, 1998) and Pectasol-C (5-10 kDa), respectively (Morris et al 2013).

POS represent the oligosaccharide obtained by pectin depolymerization. This term includes oligogalacturonides (OGalA), alpha-galactooligosaccharides (GalOS), arabinooligosaccharides (AraOS), rhamnogalacturonoligosaccharides (RhaGalAOS), xylooligogalacturonides (XylOGalA) and arabinogalactooligosaccharides (AraGalOS). These compounds are obtained after the breakdown of the various pectin fractions (HG, RG-I, RG-II) from both agro-industrial by-products and purified pectins, by enzymatic hydrolysis, acid hydrolysis, hydrothermal processing, dynamic high-pressure microfluidization or photochemical reaction in media containing TiO_2 (Gullón et al. 2013).

Regarding their bioactivity, there is a vastly amount of studies that support POS as a new class of prebiotics. Their effect on health-promoting includes, apart from being fermented by intestinal bacteria producing SCFA, stimulation of apoptosis in human colonic adenocarcinoma cells, potential for cardiovascular protection *in vivo*, reduction of damage by heavy metals, antiobesity effects, antitoxic, antiinfection, antibacterial, and antioxidant properties (Gómez et al. 2016; Babbar et al. 2016; Samuelsson et al. 2016; Maxwell et al. 2015; Gullón et al. 2013; Olano-Martín et al. 2002).

1.2.4 Metabolism and colonic fermentation of prebiotics

According with previous sections, prebiotics benefits to host health are driven mainly by two mechanisms;

The first one is associated with the positive modification of microbial population, increasing health-promoting organisms such as bifidobacteria and lactobacilli and, in certain situations, reducing the numbers of bacterias which may have a harmful role in host health (clostridia, bacteroides, enterococci, and enterobacteria) (Roberfroid et al. 2010) (**Figure 6**). In this sense, despite the large amount of publications on prebiotic properties, there is still a poor understanding of the mechanisms by which they could selectively stimulate the growth of only some members of microbial population. One example of prebiotics action is that certain members of colonic microbiota possess cell-associated glycosidases, which allow the hydrolysis of prebiotics, and the subsequent uptake of the monosaccharides released. A second paradigm is that certain microbes possess oligosaccharide transport systems that can scavenge oligosaccharides from the gut environment for the subsequent intracellular hydrolysis (Rastall 2010).

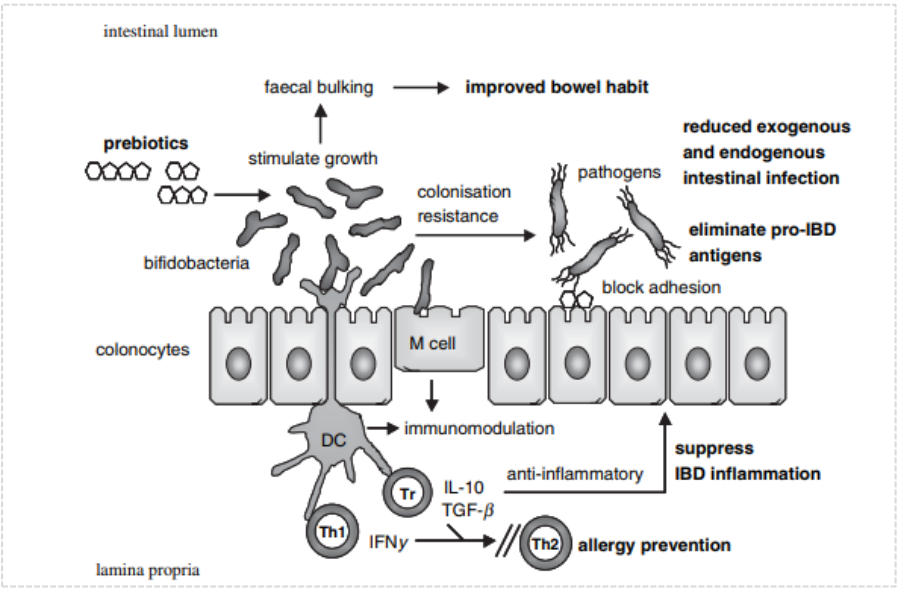

Figure 6. Postulated mechanisms of health benefits induced by prebiotics via the selective proliferation of beneficial populations of intestinal bacteria. DC, dendritic cell; Th1, T helper cell type 1; Th2, T helper cell type 2; Tr, regulatory T cell. Figure reported from Crittenden (2006) Reproduced with kind permission from Wiley.

The second mechanism of prebiotics' effect on health is related to the modifications of the metabolic activity of the microbiota based on the ability of some bacterial species to ferment prebiotic carbohydrates (saccharolytic fermentation). In this regard, the metabolization of these prebiotics by colonic bacteria is the perfect example of the synbiotic relationship between the host and the microbiota. Mammalian genomes do not encode most of the enzymes needed to degrade the structural linkages of polysaccharides present in plant material. In fact, human enzymes are capable of degrading only a few glycosidic linkages via the action of pancreatic amylase and the disaccharidases present in the brush border. Therefore, degradation of these

carbohydrates relies on the colonic bacteria that possess many different enzymes that allow them to metabolize different complex carbohydrates (Martens et al. 2011; Flint et al. 2012).

The products of saccharolytic fermentation are, principally, short chain fatty acids (SCFA) (acetate, propionate, and butyrate) as well as other products (lactate, pyruvate, ethanol, succinate) and gases (carbon dioxide, methane, hydrogen). SCFA play a crucial role for intestinal and host health and have been identified as modulators of certains aspects of metabolic activity including colonocyte function, gut homeostasis, energy gain, the immune system, blood lipids, appetite, renal physiology (Kieffer et al. 2016; Pluznick et al. 2016) and have also shown to exert a protective role against colon cancer (Bailón et al. 2010; Zeng 2014) (**Figure 7**). In this sense, a healthy microbiota is considered when saccharolytic fermentation is predominant compared to proteolytic fermentation (metabolism of peptides and proteins) that can generate toxic substances (i.e. biogenic amines and sulphides, ammonia, thiols, indols) and increase the risk of colon cancer.

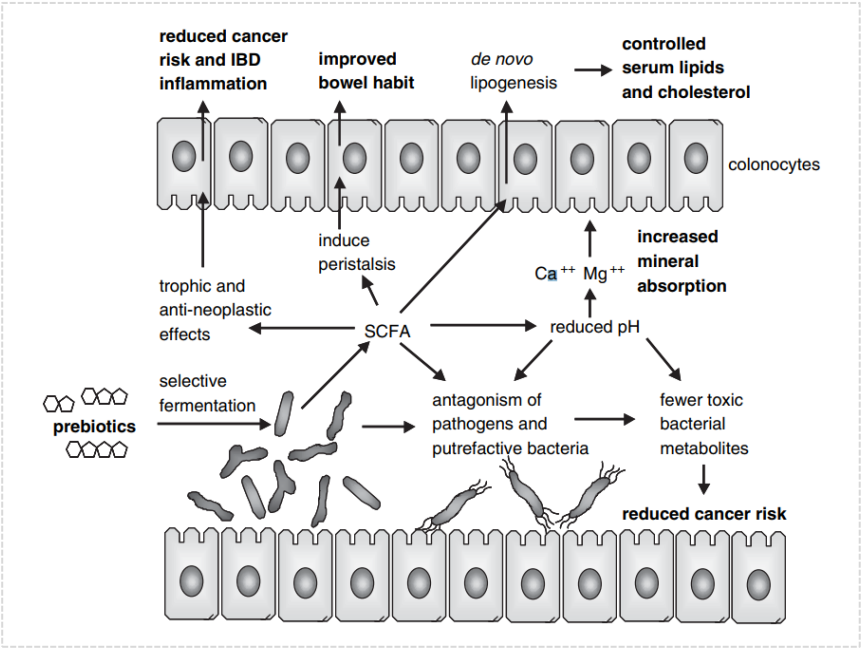

Figure 7. Postulated mechanisms of health benefits induced by prebiotics via the stimulation of beneficial microbial activities within selected populations of intestinal bacteria. IBD, inflammatory bowel diseases; SCFA, short-chain fatty acids. Figure reported from Crittenden (2006) Reproduced with kind permission from Wiley.

Furthermore, interaction between bacteria must be taken into account when studying the mechanisms of action of prebiotics. Thus, while certain bacteria such as bifidobacteria do not produce butyrate, they can however stimulate other butyrate-producing bacterial species that can use acetate and lactate (intermediate products) produced by bifidobacteria to produce butyrate (cross-feeding interaction).

However, SCFA production of itself can not be accepted as validated biomarkers of prebiotic activity, that is selected bacterial growth or activity.

Hence, well-designed methods with specific bacterial enumeration are highly preferred (Roberfroid 2005).

1.2.4.1 Colonic fermentation models

In vivo animal studies and human trials provide the most physiologically relevant information about the dynamic microbial processes during the fermentation occurring in the gastrointestinal tract. However, drawbacks such as the high costs, high complexity, inter-individual variations and the lack of easy access to the gut to sampling, restrict these *in vivo* studies to *in vitro* approaches that cannot fully provide information on dynamic microbial process in the gut (Venema et al, 2013; Verhoeckx et al. 2015). However, *in vitro* models offer unique advantages such as their reproducibility, simplicity, cost-effectiveness and the better control of the experimental variables than animal or human studies. Thus, regarding the ability of prebiotics to exert their effect on the host microbiota, a variety of *in vitro* fermentation models have been developed (Payne et al. 2012; Rumney et al. 1992). One of the simplest ways to support the fermentation on gut microbiota is the demonstration that these compounds are metabolized when incubated with pure probiotic strains in a basal medium. However, this does not confirm the selectivity of the substrate since possible interactions between bacteria are not considered. Therefore, modifications of this method

involve the use of defined mixed- or co-cultures, which introduces competition between microbes, although it still does not represent the complex interactions in the human large intestine. A more significant method to assure this complex diversity involves the use of faecal inocula, which provides a better representation of the events occurring in the distal colon. In this sense, few authors revealed that *in vitro* models can maintain a human-like gut microbiota (Rajilic-Stojanovic et al. 2010; Van den Abbeele et al. 2010), reporting that the microbial spectra unique to specific human subjects can be maintained *in vitro.* In addition, when comparing microbial fermentation spectra of different prebiotic compounds in different *in vitro* models for the human GIT, similar behaviour was observed for each prebiotic supporting the reproducibility between different *in vitro* models (Van den Abbeele et al., 2013). Besides that, results from human clinical trials have shown that bifidogenic as well as butyrogenic effect of prebiotics can be reproduced *in vitro* (Venema et al. 2003). Thus, to date, there are different *in vitro* models developed for the evaluation of the effect of prebiotics in microbiota and are briefly summarized below with their advantages and limitations (**Table 4**).

Table 4. Current available *in vitro* fermentation models with their advantages and limitations. Adapted and modified from Payne et al. (2012).

Models	Advantages	Limitations	References
Batch cultures - Single stage reactors	Easy to set up, useful for fermentation studies and especially substrate digestion assessment.	Short-term fermentation studies and weakness in microbiological control	Pompei et al. (2008); Gumienna et al. (2011)
Continuous cultures – 3-stage continuos systems	Continuous flow mimicking conditions found *in vivo*. Environmental parameters are well controlled.	No host functionality and experiments are time limited (days or weeks)	Maccaferri et al. (2010); Duncan et al. (2009); Gibson et al. (1988); Macfarlane et al. (1989)
Multistage continuous cultures – 4-5 stage system	Continuous flow into several vessels mimicking conditions found in portions of the digestive tract.	No host functionality and experiments are time limited (days or weeks)	Minekus et al. (1999); Mäkivuokko et al. (2005); Van den Abbeele et al (2010); Barroso et al. (2015)
Immobilized continuous cultures	High-cell density and long-term stability of continuous fermentation system with immobilized faecal microbiota.	No host functionality	Le Blay et al. (2009); Zihler et al. (2010)
Artificial digestive systems	Continuous flow with metabolites and water exchange mimicking conditions found *in vivo*	No immune and neuroendocrine response and experiments are limited to few days' time	Blanquet-Diot et al. (2009); Kovatcheva-Datchary et al. (2009)

In this regard, development of multistage continuous fermentation models, so-called "gut models" which enable the simulation throughout the entire colon processes by setting up different vessels in series and the artificial digestive systems represent the most advanced attempt, so far, at

simulating interdependent physiological functions within the human gut, stomach and small intestine (Verhoeckx et al, 2015). Therefore, these i*n vitro* models allow a dynamic sampling over time in the consecutive regions of the gut providing a unique tool to study the possible mechanism of action of dietary ingredients or drugs. To date, few multistage continuos models have been developed and provide probably the closest simulation to *in vivo* conditions (Molly et al. 1993; Minekus et al. 1995; Minekus et al. 1999; Barroso et al. 2015; Mäkivuokko et al. 2005; Cinquin et al. 2004). However, although dynamic mulstistage models also facilitate long-term studies, they can present disadvantages such as, they are expensive to set up, take more labor intense and time consuming and require higher operation costs in terms of working volumes and addition of substances mimicking gastrointestinal fluids. Additionally, *in vitro* continuos systems may oversimplify *in vivo* situation due to the absence of metabolits/substrates absorption in some of these models. It is therefore difficult to properly reproduce *in vitro* all entirely colon fermentation dynamics although high valuable information can be obtained due to their use (Macfarlane & Macfarlane 2007). Thus, given the high interest on this topic, important new approaches have being developed in the last decade, such as, the combination of dynamic gut models with human cell culture systems (Bahrami et al. 2011; Marzorati et al. 2014) or the cultivation of single intestinal stem cells into spherical crypt-like structures,

called “organoids”, with several distinct cell types found in the gut (Roeselers et al. 2013; Sato et al. 2009), among other approaches (Barrila et al. 2010; Radtke et al. 2010)

1.3 Resistance to the gastrointestinal digestion of prebiotic carbohydrates

As indicated above, one of the requirements for these compounds to be considered as prebiotics is their resistance to the upper gastrointestinal tract digestion. In this sense, ever since prebiotics were first defined in 1995, the investigations have focused on their effect on the microbiota activity and/or composition. 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. Whether or not chemical or structural changes occur when they are exposed to this environment is important, because even minor chemical or structural differences could substantially affect their properties and therefore their impact on colonic microbiota (Li et al. 2015).

Human gastrointestinal degradation of carbohydrates constitutes a multistage process starting in the oral cavity with a very scarce hydrolysis by the α-amylase present in the saliva and continues its degradation and

absorption in the small intestine, being the main site for carbohydrate digestion involving the secreted pancreatic α-amylase. 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. In fact, dietary carbohydrate digestion involves up to six different carbohydrases produced by three different organs (**Table 5**). Moreover, the mammalian upper gastrointestinal tract (small intestine) contains a variety of distinct microbial population. More acidic conditions and higher levels of oxygen than those present in colon allow a dominance by fast-growing facultative anaerobes, such as Lactobacillaceae or Enterobacteraceae, that tolerate the combined effect of antimicrobials and bile salts (Donaldson et al. 2016). In this regard, conditions such as, presence of oxygen, antimicrobial compounds (bile salts) or pH, limit the bacterial density below 10^4 colony-forming units (cfu/g) (**Figure** 1) 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 can suffer changes that could affect their properties when they reach the colon to be fermented.

Table 5. Human carbohydrases involved in dietary carbohydrate digestion. Reproduced from Hernandez-Hernandez et al. (2019).

Digestive carbohydrases	Type of enzyme	Glycoside Hydrolase Family[1]	Production organ / Main site of digestion	Glycosidic linkage specificity	Main substrates[2]	Main products[2]
Salivary α-amylase[3]	Secreted (α-glucosidase)	13	Salivary gland / Mouth	Glcα(1→4)Glc	Starch; linear maltooligosaccharides (n>6)	Maltose; maltotriose; α-dextrins
Pancreatic α-amylase[3]	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-phorizin 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

[1] According to CAZy database (http://www.cazy.org/) [Lombard et al. (2013)].
[2] Based on and updated from Alpers (2003).
[3] Human salivary and pancreatic amylases have 94% amino acid identity although they are encoded by different genes. [Meisenberg & Simmons (2016)]

1.3.1 Models for the assessment of digestibility

To date, only scarce and fragmented information on their pass throughout the small intestine is available. As it is well-known, *in vivo* feeding methods, using animals or humans, usually provide the most accurate results but they are time consuming and very costly, that is why much effort has been devoted to the development of *in vitro* procedures. Few *in vivo* studies have evidenced the partial degradation of prebiotic compounds after their pass through the small intestine. Holloway et al. (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 90% of recovery in the terminal ileum after colonic intubation of volunteers. Moreover, Hernandez-Hernandez et al. (2012) found out 13-53% of small intestinal digestibility of purified GOS (with a degree of polymerization from 3 onwards) in an *in vivo* study with rats.

Apart from that, *in vitro* digestion models provide a very useful alternative to animal and human models by rapidly screening food ingredients. In this sense, the most frequently approach to measure the digestion process is by simulating as much as possible the human physiological conditions, taking into account the presence of digestive enzymes and their concentrations, pH, temperature, digestion time, and salt

concentrations, among other factors (Kopf-Bolanz et al. 2012; Minekus et al. 2014).

Static methods represent the simplest techniques in this context and could include two or three separated digestion steps (oral, gastric and intestinal). 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, significant compound degradation from food samples is not expected in this stage. However, although the majority of models reported in literature are static simulators, some computer-controlled multi-compartmental continuous system models (**Table 4**) overcome some limitations present on static models allowing the simulation of dynamic aspects of digestion, such as transport of digested components, variable enzyme concentrations, pH changes, peristaltic movements, continuous changes, and secretion flow rates (Ouwehand & 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 (Barroso et al. 2015; Minekus et al. 1995; Molly et al. 1993).

1.3.1.1 Standarised protocols of digestion

The most widely accepted standardised static *in vitro* method for digestion was developed in 2014 (Minekus et al. 2014). The static protocol published by InfoGest network represents an international consensus of scientist from 32 countries working in the field of digestion and it has been widely used in several works since its release (Egger et al. 2016). Given the large variety of methods available, this *in vitro* digestion consensual method for food aimed to unify and produce data that are more comparable and reproducible between studies (**Figure 8)**.

This developed method presents two approaches regarding the enzymes used at the small intestine stage. Firstly, the use of a pancreatic extract (pancreatin) containing all the relevant enzymes is suggested for reasons of simplicity. As alternative, individual enzymes such as trypsin, chymotrypsin, pancreatin lipase, colipase, pancreatin amylase and bile salts can also be used. However, given the complexity of the gastrointestinal process at the small intestine, enzymes and substrates proposed in this method for intestinal digestion cannot reflect the real enzymes activities of the human gut towards carbohydrate digestion since the model ignores the inclusion of small intestinal mucosal carbohydrases (**section 1.3** and **Table 5**).

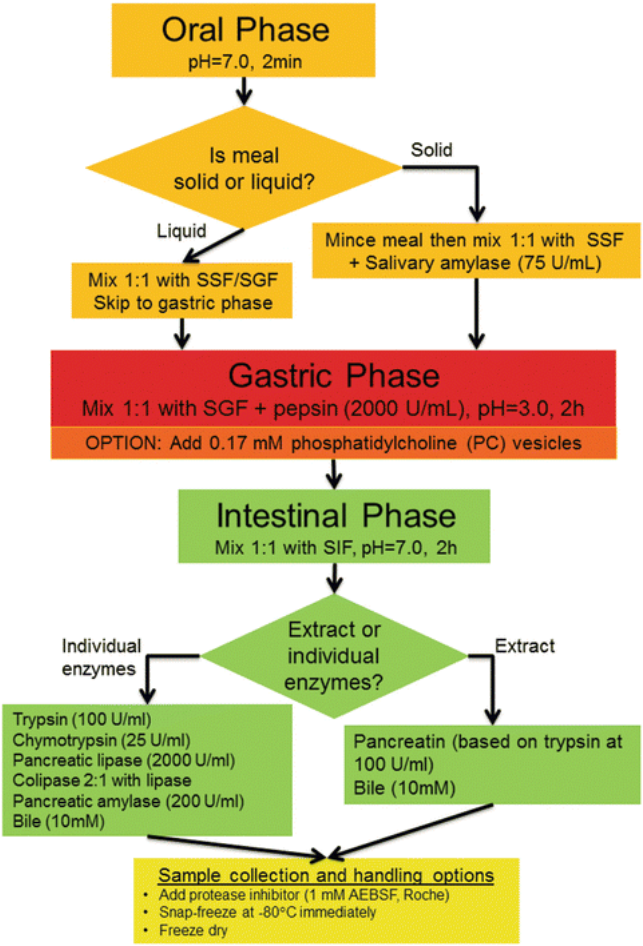

Figure 8. Flow diagram of the digestion presented at the InfoGest Consensus method involving simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Figure reproduced from Verhoeckx et al. (2015) Chapter 2 (Mackie & Rigby) with kind permission of Springer.

Based on the literature, only few studies have been carried out on the resistance to digestion of non-digestible carbohydrates. Most of them describe this process as a previous step for the latter fermentation of these compounds and no focus is put on their digestibility, assuming they reach the colon without alterations. As it has been said above, the most common

approach to simulate the small intestinal digestion is by the use of digestive enzymes from pancreas, which could not really reflect the enzyme activity of the upper gastrointestinal digestion. However, studies on the digestion of different types of fibre such as resistant starch, FOS, pectic polysaccharides from kiwifruit, and sugar beet pectin using simulated gastric and intestinal fluids, similar to InfoGest method, have shown a partial degradation or changes in their structural features (Foucault et al. 2016; Sancho et al. 2017; Logan et al. 2015; Carnachan et al. 2012). Molecular weight diminution and decreases in their initial concentration were observed on these substrates, although 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 Chemist (AOAC) developed an integrated determination method for dietary fibre, including non-digestible oligosaccharides and resistant starch (AOAC 2009.01) (McCleary et al 2010). This method, as well as the others, is based on the use of isolated digestive enzymes. Porcine pancreatic α-amylase 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, similar to the InfoGest protocol, these

enzymes cannot completely hydrolyse digestible saccharides such sucrose, lactose, panose, etc. since they not represent the fully complex enzymatic environment of the small intestine. As a result, digestible saccharides that are not fully degraded are detected as non-digestible oligosaccharides leading to an inaccurate determination of these resistant carbohydrates (Tanabe et al. 2014).

Consequently, alternative methods based on the use of mammalian intestinal extracts, which could provide a more accurate approach of the intestinal process, represent a good option to overcome the disadvantages of the use of isolated enzymes. In this sense, similarity between humans and rats with regard to hydrolysing 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. To date, there are limited reports regarding the use of a rat small intestinal extract for the evaluation of digestibility of non-digestible carbohydrates. The most common use of these extracts is referred to previous steps of digestion focusing on their fermentability, (Kaulpiboon et al. 2015; Ito et al. 2008). However, other studies have also successfully applied these

intestinal enzymes from rat for the assessment of digestibility of resistant carbohydrates, such as FOS (Oku et al. 1984), GOS (Ohtsuka et al. 1990), as well as to a range of maltose and sucrose isomers (Lee et al. 2016).

Furthermore, 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 et al. (2014). In this sense, incomplete degradation of digestible saccharides that lead to overestimate non-digestible oligosaccharides was improved by the addition of the enzymes present at the small intestinal brush border from pig, providing therefore a more realistic environment and an accurate quantification method of non-digestible oligosaccharides in processed food (Tanabe et al. 2015).

Nonetheless, use of these extracts still remains sparsely used despite of they have proved their successful utility on carbohydrate digestion and their similarity to human intestinal activity (Oku et al. 2011; Humphray et al. 2007; Lander et al. 2001). Therefore, the lack of interest on the intestinal degradation of non-digestible carbohydrates, has led to the use of standardized official methods to determine their digestibility despites their limitation (Drechsler et al. 2018; Egger et al. 2016; McCleary et al 2010). Moreover, the scarce information about the use of mammalian intestinal enzymes highlights the need for developing a standardised method that may

provide a better understanding of the processes occurring in the small intestine and, so, that it can be used in the evaluation of functional ingredients in food.

JUSTIFICATION AND AIM OF THE RESEARCH

2. Justification and aim of the research

In last decades, the interest towards the modulation of the human microbiota has undergone a huge growth due to its strong relationship with the regulation of the health and well-being. To date, several illnesses such as, celiac disease, obesity, colorectal cancer, irritable bowel syndrome, liver and cardiovascular pathologies, bone ailment and, in recent years, several neurological/psychiatric disorders have been also related with the incorrect function or alterations in the composition of the human microbiota. One of the most common used approaches to regulate the homeostasis, maintaining a proper functioning/behaviour 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 systemic level. Thereby, since prebiotics were first defined in 1995, relevant advances have been made on the development of potential new prebiotic compounds. In this regard, one of the focuses has pointed toward the obtainment of molecules with new structures of higher molecular weight as is the case of the oligosaccharides derived from lactulose, which could reach the distal portions of the gut where the main chronic diseases can take place. Another approach that has been carried out in recent years is the search for new sources of potential prebiotics that can involve more efficient, sustainable, simple and

less expensive obtainment processes. That is the case of pectin and pectic derivatives whose obtainment from waste by-products from the agricultural industry represents a very interesting alternative for this emerging prebiotic.

However, despite the great progress that has been made related to prebiotics and their benefits on human health, few studies have been made concerning their changes during their passage in the upper gastrointestinal tract, even when structural differences/alterations could substantially affect their properties. Nevertheless, the scarce information on this regard and the lack of specific methods to accurately determine their digestion, compel the use of general standardized methods/protocols such as the AOAC or InfoGest methods, which cannot reflect the real enzymes activities during the human small intestinal digestion of carbohydrates.

Therefore, taking the above indicated as starting point, this Thesis aims to contribute to provide more insight into the gastrointestinal digestion and degradation that recognized and emerging prebiotics could undergo in the upper gastrointestinal tract, and to support the potential prebiotic properties of the latter obtained from waste by-products from the agro-food industry. Therefore, this Thesis comprises a multidisciplinary study that includes: i) a more suitable method to evaluate digestibility of carbohydrates using mammalian small intestinal extracts; ii) a wide range of analytic

techniques and iii) the use of robust fermentation *in vitro* models for the evaluation of the bioactivity of substrates. In this sense, the main objectives of this PhD Thesis are **the evaluation of the *in vitro* digestibility of prebiotics such as lactulose, galactooligosaccharides (GOS) and fructooligosaccharides (FOS) using mammalian small intestinal mucosal carbohydrases and the *in vitro* fermentability of emerging proposed prebiotics such as pectin and derivatives**. Hence, in order to accomplish these two main objectives, the following partial objectives have been established:

1. ***In vitro* digestibility of dietary prebiotics using mammalian digestive enzymes.**

a) To characterize (enzymatic activity, protein content, monosaccharide composition) a commercial small intestinal extract from rats and to establish the adequate conditions to carry out a simulated *in vitro* digestion of carbohydrates. To meet that aim, temperature and the ratio carbohydrate:enzyme has been optimized.

b) To validate the *in vitro* digestion method developed evaluating the digestibility of dietary carbohydrates (lactose, sucrose, and maltose) and prebiotics with different structure such as lactulose, GOS with different linkages and monomers and lactosucrose. Degradation was

followed using adequate analytic techniques by the measurement of the release of their monomers and the diminution of initial concentrations after digestion.

c) To perform a standardized *in vitro* gastrointestinal digestion model (InfoGest) on the digestion of prebiotics added to a food matrix such as milk and to compare the different effect of the gastrointestinal digestion with the *in vitro* digestion model obtained with the small intestinal rat extract.

d) To apply an *in vitro* method using the brush border membrane vesicles from the small intestine of pig, evaluating the impact of different structural features of GOS in their capability to resist enzymatic degradation. All of this has been performed to establish the structure-function relationships of these oligosaccharides by using small intestinal mucosal carbohydrases.

2. ***In vitro* digestibility and fermentability of pectin and pectic compounds obtained from agricultural by-products.**

e) To structurally characterize (degree of polymerization, monosaccharide composition, degree of methoxyl esterification) a range of modified pectins obtained by enzymatic hydrolysis of initial pectin. Once the structure was elucidated, an *in vitro* evaluation of the

fermentation properties was carried out in pH-controlled batch fermentation systems inoculated with human faecal slurries.

f) To study the behaviour of pectin during its gastrointestinal digestion and fermentation properties in a dynamic multi-compartmental continuous *in vitro* model, which simulated the process occurring in the stomach, small intestine, and ascending, transverse and descending colon.

Work plan and overview of the research

3. Work Plan and structure/outline of the Thesis

In order to achieve the goals outlined above, the work plan for this PhD Thesis is schematically represented below. Thus, the schematic diagram provides an overview of the studies carried out which have been organized in two main parts.

On a first stage, the enzyme activity characterization of a commercial small intestinal extract from rat was carried out for its subsequent application to the digestibility of different recognized prebiotic and dietary carbohydrates (*Chapter 1)*. Successful optimization of the method allowed its application in the assessment of the matrix effect during the degradation of prebiotic within a real food such as milk, considering a first step of gastrointestinal digestion by InfoGest method (*Chapter 2)*. In addition, non-commercial intestinal brush border membrane vesicles isolated in the laboratory from pig whose carbohydrase activities were firstly tested, was also used to, eventually, determine the effect of structural features of prebiotics on their intestinal digestion (*Chapter 3)*.

On a second part, a subset of different pectins and modified pectins obtained from agro-food wastes were subjected to a static *in vitro* fermentation to evaluate their potential prebiotic properties (*Chapter 4*). Lastly, given the potential prebiotic properties observed in the static

fermentation, a commercial pectin isolated from citrus by-products was subjected to an *in vitro* dynamic gastrointestinal system (*Chapter 5*). A multi-compartmental simulator (simgi®) was used at this stage, evaluating the changes on pectin structure during digestion and the positive effect on microbiota.

Each of these chapters corresponds to scientific papers (all of them published in peer-reviewed journals) that this PhD Thesis has triggered. They are presented in the conventional format of publication (abstract, introduction, material and methods, results and discussion, and conclusion). The abstract briefly states the purpose of each research, the most-remarkable results and major conclusions. Introduction states the objectives of the work and provides an adequate background to the article. Material and methods section shows the details about the starting material and the methods to allow the work to be reproduced. A results and discussion section (combined or not) explores the significance of the results of the research. Finally, the main conclusions of the study are presented in a short conclusions section, which may stand alone or form a subsection of a results and discussion section. References of all chapters are placed at the end of the manuscript. In addition, reader can find supplementary material to chapters when it is specified at the end of the dissertation (*Annexes A-C),* an additional published scientific paper

carried out in collaboration with a private enterprise of digestive supplements (*Annex D*) and the scientific publications obtained from the work developed in this Thesis (*Annex E).*

In vitro digestibility and fermentability of selected prebiotics and functional carbohydrates with prebiotic potential

Part I

In vitro digestibility of dietary prebiotics using mammalian digestive enzymes

Chapter I

Development and Validation of an *in vitro* small intestinal digestion model

- Characterization of a commercial intestinal extract of rat
  - ✓ Enzymatic activity, protein content
- Optimization of reaction conditions
- *In vitro* digestion of recognised prebiotics and dietary carbohydrates
- GC-FID monitoring of carbohydrates degradation

Chapter II

In vitro digestion of prebiotics and effect of a food matrix

- Digestion of prebiotics within a food sample (milk)
  - ✓ InfoGest method (oral, gastric, intestinal phase)
  - ✓ Mammalian intestinal extract (intestinal phase)
- Monitoring of the effect of carbohydrates on protein digestion
- Differences between digestion at the intestinal phase

Chapter III

Application of the proposed method with other mammalian extracts

- Obtainment and characterization of non-commercial intestinal brush border membrane vesicles from pig
- *In vitro* digestion of recognised prebiotics
- Establishment of structure-function relationship

Part II

In vitro digestibility and fermentability of pectin and pectic compounds obtained from agricultural by-products

Chapter IV

In vitro bioactivity of potential prebiotic compounds obtained from waste by-products

- Enzymatic obtainment and structural characterisation of modified pectins from sunflower, artichoke and citrus pectin
  - ✓ Polymerization degree, monosaccharide composition, methoxylation degree
- Faecal batch cultures
  - ✓ Changes in microbial population (FISH)
  - ✓ SCFA production

Chapter V

In vitro evaluation of the behaviour of pectin during the gastrointestinal digestion and intestinal fermentation

- Multistage dynamic gastrointestinal simulator (simgi®)
  - ✓ Gastric, small intestinal, ascending, transverse and descending colon stages
- Molecular weight changes after gastric and intestinal digestion
- Fermentation in colonic stages
  - ✓ Changes in microbial population
    - ➢ Plate counts
    - ➢ *q*PCR
  - ✓ SCFA production
  - ✓ Ammonium production

RESULTS AND DISCUSSION OF THE RESEARCH

4. Results and Discussion

Part I: *In vitro* digestibility of dietary prebiotics using mammalian digestive enzymes

Chapter 1: Assessment of *in vitro* digestibility of dietary carbohydrates using rat small intestinal extract

Chapter 2: Study on the digestion of milk with prebiotic carbohydrates in a simulated gastrointestinal model

Chapter 3: *In vitro* digestibility of galactooligosaccharides: Effect of the structural features on their intestinal degradation

Chapter 1

Assessment of *in vitro* digestibility of dietary carbohydrates using rat small intestinal extract

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Abstract:

There are few studies on the assessment of digestibility of non-digestible carbohydrates, despite their increasingly important role in human health. In vitro digestibility of a range of dietary carbohydrates classified as digestible (maltose, sucrose, and lactose), well recognized (lactulose, fructooligosaccharides (FOS), and two types of galactooligosaccharides (GOS) differing in the predominant glycosidic linkage), and potential (lactosucrose and GOS from lactulose, OsLu) prebiotics using a rat small intestinal extract (RSIE) under physiological conditions of temperature and pH is described. Recognized and potential prebiotics were highly resistant to RSIE digestion although partial hydrolysis at different extents was observed. FOS and lactulose were the most resistant to digestion, followed closely by OsLu and more distantly by both types of GOS and lactosucrose. In GOS, $\beta(1\rightarrow 6)$ linkages were more resistant to digestion than $\beta(1\rightarrow 4)$ bonds. The reported in vitro digestion model is a useful, simple, and cost-effective tool to evaluate the digestibility of dietary oligosaccharides.

Introduction:

There is growing evidence indicating that dietary nondigestible oligosaccharides (NDO) play an increasingly important role in health. Low glycemic index foods, characterized by slowly absorbed carbohydrates, are linked with reduced risk of common chronic Western diseases associated with central obesity and insulin resistance (Jenkins et al. 2002; Augustin et al. 2002). These pieces of evidence have boosted the interest in the use of nondigestible (or with slow digestion rate) carbohydrates as food ingredients due to their ability to reduce postprandial glycemic response (Lee et al. 2016). The attention to NDO is also reinforced by the fact that regulatory agencies such as the EFSA have acknowledged that the consumption of foods/drinks, in which NDO replace simple sugars, reduces postprandial glycaemic and insulinaemic responses. This behavior is attributed to the resistance of NDO to hydrolysis and absorption in the small intestine (EFSA 2011; EFSA 2014a, 2014b, 2014c).

A specific subset of nondigestible carbohydrates, so-called prebiotics, have attracted especial interest due to their capability to reach the colon and be selectively fermented by the intestinal microbiota that results in specific changes in its composition and/or activity, thus contributing to human health promotion (Gibson et al. 2004; Roberfroid et al. 2010). Intestinal microbiota

plays an important role in a great variety of physiological process, such as the development of the host immune system, anti-inflammatory activity, uptake of energy from the host diet, production of short-chain fatty acids by fermentation, alteration of human glucose and fatty acid metabolism, regulation of intestinal permeability, or stimulation of mineral absorption by the large intestine (Chung et al. 2010; Ding et al. 2010; Havenaar 2011; Giacco et al. 2016).

However, despite the generally accepted concept that NDO pass through the upper gastrointestinal tract without substantial modifications (Roberfroid et al. 2010), few efforts have been made toward the study of the resistance of this type of oligosaccharides to the digestion in the small intestine, and only scarce and fragmented information on their pass throughout the small intestine is available. In this context, the limitations of AOAC method 2009.01 (McCleary et al 2010), for the measurement of NDO have already been highlighted, such as the use of a very limited number of enzymes (i.e., α-amylase and amyloglucosidase) which fail to hydrolyze digestible saccharides (including sucrose or starch-decomposed products), as well as the use of enzymes from fungal origin despite it being well-known that the hydrolyzing activity of enzymes from fungal or microbial sources does not reflect the carbohydrase activities of enzymes of the human

gastrointestinal tract.(Tanabe et al. 2014). Consequently, alternative methods, which are based on the use of mammalian intestinal enzymes, such as those derived from pigs (Tanabe et al. 2015) and weaning piglets, (Strube et al. 2015) have recently been proposed. However, up to date, the regular supply of porcine small intestinal enzymes is not commercially available, which may hinder an easy and broad implementation of these useful methods to evaluate the in vitro intestinal digestion of oligosaccharides. In this sense, the use of rat small intestinal extract (RSIE) can be advantageous because of its commercial availability, as well as the reported similarity of hydrolyzing activities between human and rat small intestinal disaccharidases (Oku et al. 2011). In fact, the use of RSIE has been successfully applied for the assessment of digestibility of prebiotics, such as fructooligosaccharides (FOS) (Oku et al. 1984) and galactooligosaccharides (GOS), (Ohtsuka et al. 1990; Ferreira-Lazarte et al. 2017a) as well as to a range of maltose and sucrose isomers3 and isomaltooligosaccharides (Kaulpiboon et al. 2015). However, to the best of our knowledge, a comparative study of well-recognized prebiotics, that is FOS, GOS, and lactulose, and potential and novel candidates, such as lactosucrose and GOS derived from lactulose (OsLu), has not been carried out. Particularly, GOS comprise a complex mixture of, mainly, disaccharides and trisaccharides having a variety of glycosidic linkages with β-anomeric configuration. While $\beta(1\rightarrow4)$ and

β(1→6) are the most common glycosidic linkages found in GOS structures, *β*(1→2) and *β*(1→3) can also be found in the mixture (Torres et al. 2010; Otieno et al. 2010). Bearing in mind that there is evidence in the literature indicating that the linkage type could be a factor more important than monomer composition in determining the susceptibility of carbohydrates to digestive glycosidases (Lee et al. 2016), the enzymatic susceptibility of GOS could be largely affected by differences in the predominant glycosidic linkage.

Therefore, the aim of the present study was to evaluate the small intestinal digestibility of well-recognized prebiotics, that is lactulose, FOS (kestose and nystose), and two types of conventional GOS with predominant β(1→4) or β(1→6) linkages, respectively, as well as emerging prebiotic candidates such as lactosucrose and OsLu, and their comparison with digestible disaccharides (lactose, sucrose, and maltose) used as appropriate controls in an in vitro digestion model using RSIE.

Materials and methods:

Chemicals and reagents. Fructose (Fru) standard was purchased from Fluka analytical. D-Galactose (Gal), D-glucose (Glc), lactose (*β*-D-Gal(1→4)-D-Glc), sucrose (*β*-D-Fru(2→1)-*α*-D-Glc), maltose (α-D-Glc(1→4)-D-Glc), trehalose (*α*-D-Glc(1→1)-*α*-D-Glc), palatinose (also termed isomaltulose) (*α*-

D-Glc(1→6)-D-Fru), lactulose (*β*-D- Gal(1→4)-D-Fru), kestose (*β*-D-Fru(2→1)-*β*-D-Fru(2→1)-*α*-D-Glc), nystose (*β*-D-Fru(2→1)-*β*-D-Fru(2→1)-*β*-D-Fru(2→1)-*α*-D- Glc), phenyl-β-glucoside, o-nitrophenyl (*o*-NP), *p*-nitrophenyl (*p*-NP), *o*-nitrophenyl-*β*-D-glucopyranoside (*o*-NPG) and *p*-nitrophenyl-*α*-glucopyranoside (*p*-NPG) standards, and intestinal acetone powders from rat (Rat Small Intestinal Extract, RSIE) were obtained from Sigma-Aldrich (St Louis, MO). Lactosucrose (*β*-D-Gal(1→4)-*α*-D-Glc(1→2)-*β*-D-Fru) standard was obtained from Wako Chemical Industries (Neuss, Germany). All standard carbohydrates were of analytical grade (purity ≥ 95 %).

Obtainment of prebiotic ingredients. OsLu were obtained at pilot scale by the company Innaves S.A. (Vigo, Spain) following the method described by López-Sanz et al. (2015). In brief, OsLu were synthesized using a commercial lactulose preparation (670 g/L; Duphalac, Abbott Biologicals B.V., Olst, The Netherlands), diluted with water at 350 g/L and pH adjusted to 6.7 with KOH, and a *β*-galactosidase from *Aspergillus oryzae* (16 U/mL; Sigma). The mixture of oligosaccharides (20% [w/v]) was treated with fresh *Saccharomyces cerevisiae* (1.5% [w/v]; Levital, Paniberica de Levadura S.A., Valladolid, Spain) at 30 °C and aeration at 20 L/min to remove monosaccharides. Finally, the samples were vacuum concentrated at 40 °C in

a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). The two commercial GOS syrups with predominant $\beta(1\rightarrow4)$ (named GOS-1) and $\beta(1\rightarrow6)$ (named GOS-2) linkages were kindly provided by the corresponding manufacturers whereas a mixture of FOS consisting of kestose and nystose was obtained from Wako Chemical Industries (Neuss, Germany).

The composition of OsLu syrup, whose main involved glycosidic linkage was $\beta(1\rightarrow6)$, expressed in g per 100 g of ingredient was as follows: 0.5% fructose, 12.5% galactose, 26% lactulose, 19.6% OsLu disaccharides, 16.0% OsLu trisaccharides (making 61.6% of potential NDO) and 25.7% moisture. GOS-1 syrup had 21% moisture and the composition of carbohydrates was 1% galactose, 21.4% glucose, 13% lactose, 19.2% GOS-disaccharides, 20.8% GOS-trisaccharides and 3.6% GOS-tetrasaccharides (equivalent to 43.6% of potential NDO). GOS-2 syrup composition was: 6.7% galactose, 22.6% glucose, 19.0% lactose, 9.9% GOS-disaccharides, 14.2% GOS-trisaccharides, 0.7% GOS-tetrasaccharides (equivalent to 24.8% of potential NDO) and 27% moisture.

Determination of protein content and main enzyme activities of the Rat Small Intestinal Extract (RSIE). RSIE was used to prepare an enzyme/enzymatic solution according to the method of Olaokun et al. (2013), with minor modifications. RSIE (10 mg/ mL) was homogenized in ice-cold

0.05 M sodium phosphate buffer solution. Then, the solution was centrifuged at 2,415 x g for 15 min and the supernatant obtained was used as the enzyme solution for determining protein content and enzymatic activity.

Protein content. The total protein content of the enzymatic solution was quantified according to the Bradford method using the Bio-Rad Protein Assay kit and bovine serum albumin as a standard. The absorbance was monitored at 595 nm (Bradford 1976).

Hydrolytic activities. ***β-galactosidase and maltase activities***. The determination of the rat intestinal *β*-galactosidase activity was adapted from Warmerdam et al. (2014). A solution of *o*-NPG in phosphate buffer 0.05 M, pH 7.0 with a concentration of 0.5 mg/mL (0.05% w/w) was prepared. The enzymatic activity was determined by incubating 1,900 µL of the o-NPG solution and 100 µL of enzyme solution from RSIE for 2 h at 37 °C. The method is based on the measuring of the continuous release of *o*-NP from *o*-NPG. Absorbance of released *o*-NP was measured at 420 nm every 20 s using a spectrophotometer (Specord Plus, Analytik Jena) together with a temperature controller (Jumo dTRON 308, Jumo Instrument Co.). The specific enzymatic activity (U) was expressed in µmol min^{-1} g^{-1}, where one unit was defined as the amount of enzyme that produced 1 µmol of *o*-NP in one min of reaction (n=6). Similar procedure was used to determine the

maltase activity but using a solution of *p*-NPG in phosphate buffer 0.05M, pH 6.8 with (0.05% w/w) and monitoring the release of *p*-NP at 420 nm every 20 s (n=3).

Sucrase, trehalase and palatinase activities. Sucrase, trehalase and palatinase activities were determined following the method described by Ghazi et al. (2005) with slight modifications. An individual solution of sucrose, trehalose or palatinose (0.5% w/w) in sodium phosphate buffer 0.05 M, pH 6.5 was used. An eppendorf tube with 250 µL of sucrose, trehalose or palatinose solution was preheated at the reaction temperature, 37 °C. Subsequently, 100 µL of enzyme solution was added and the mixture was incubated for 2 h and different aliquots were taken at different times (5, 10, 15, 30, 60, 90, and 120 min). Hydrolysis was stopped by adding 350 µL of a 3,5-dinitrosalicylic acid (DNS) solution prepared according to Asare-Brown & Bullock (1988). Sucrase, trehalase and palatinase activities were determined measuring the reducing sugars released from the corresponding disaccharide hydrolysis, at 540 nm, according to the DNS method (Miller 1959). The specific enzymatic activity (U) was expressed in µmol min^{-1} g^{-1}, where one unit was defined as the amount of enzyme that produced 1 µmol of reducing sugars in one min of reaction (n=3).

***In vitro* small intestinal digestion using RSIE**. The digestibility of two types of conventional GOS, (GOS-1 and GOS-2), OsLu, a mixture of FOS (comprised of kestose and nystose), lactosucrose, lactulose and digestible oligosaccharides such as lactose, sucrose and maltose were evaluated using RSIE. In a first step, preliminary assays aimed to determine an optimal RSIE-carbohydrate weight ratio within 2 h of reaction were carried out using lactulose and lactose as appropriate controls. Finally, a solution of 20 mg of RSIE and 1 mL distilled water was prepared as a digestive enzyme solution, resulting in a pH value of 6.8. Subsequently, 0.5 mg of carbohydrate was added and the mixture was incubated at 37 °C under continuous agitation (450 rpm) for 2 h. Aliquots were taken at 15, 30, 60, 90, and 120 min of digestion and heated in boiling water for 5 min to stop the reaction. The digestion was monitored by GC-FID as described below. In addition, a series of control samples, based on the incubation of RSIE without carbohydrates during the same reaction times, were analysed. Results showed a slight increase of galactose and a notable release of glucose as the digestion proceeded. These values were conveniently subtracted in order to avoid any overestimation of the monosaccharide fraction.

Carbohydrate analysis by GC-FID. Trimethylsilylated oximes (TMSO) of carbohydrates (mono-, di- and trisaccharides) present in samples were

determined following the method of Cardelle-Cobas et al. (2009b). Chromatographic analysis was carried out on an Agilent Technologies gas chromatograph (Mod7890A) equipped with a flame ionization detector (FID). The TMSO were separated using a 15 m x 0.32 mm × 0.10 μm film, fused silica capillary column (DB-5HT, J&W Scientific, Folson, California, USA). Nitrogen was used as carrier gas at a flow rate of 1 mL/min. Injector and detector temperatures were 280 and 385 °C, respectively. The oven temperature was programmed from 150 to 380 °C at a heating ratio of 3 °C/min. Injections were made in the split mode (1:5). The TMSO derivatives were formed following the method of Ruiz-Matute et al. (2012). First, a volume of 450 μL of the resulting intestinal digesta, corresponding to 225.0 μg of saccharides was added to 200 μL of internal standard solution, containing 0.5 mg/mL of phenyl-*β*-glucoside. Afterward, the mixture was dried at 40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). Sugar oximes were formed by adding 250 μL hydroxylamine chloride (2.5%) in pyridine and heating the mixture at 70 °C for 30 min. Subsequently, the oximes obtained in this step were silylated with hexamethyldisylazane (250 μL) and trifluoroacetic acid (25 μL) at 50 °C for 30 min (Brobst & Lott 1966). Derivatization mixtures were centrifuged at 6,700 x g for 2 min and supernatants were injected in the GC. Data acquisition and integration were done using Agilent ChemStations software

(Wilmington, DE, USA). Response factors were calculated after the duplicate analysis of standard solutions (fructose, galactose, glucose, lactose, lactulose, sucrose, raffinose and stachyose), at different concentrations ranging from 0.005 to 4 mg/ mL.

Statistics. All digestions were carried out in duplicate and two GC-FID analysis were performed for each digestion treatment (n=4). The comparisons of means using analysis of variance (ANOVA) were made using the statistical package (SPSS Inc., Chicago, Il). The differences were considered significant when $p<0.05$.

Results and discussion:

Determination of the main enzymatic activities of the RSIE. Table 1 shows the protein content and β-galactosidase, maltase, sucrose, trehalase and palatinase activities of RSIE measured under the assayed digestion conditions. Maltase activity was the highest with 17-, 19-, 65 and 134-fold increases as compared to *β*-galactosidase, sucrose, trehalase and palatinase activities, respectively. These data are in agreement with the huge difference previously reported between the activity of maltase and the rest of disaccharidases of the whole region of the small intestine of rats (Oku et al. 2011). In consequence, RSIE exhibited much more moderate β-galactosidase

and sucrase activities with similar values of 26.7 U and 23.5 U, respectively. In contrast to these values, Oku et al. (2011) observed a 4-fold increase of sucrase activity as compared to β-galactosidase activity in the small intestine. This dissimilarity could be attributed to several methodological factors, such as different assay and detection methods, and/or different substrates (lactose vs *o*-NPG) used in both studies. In addition, β-galactosidase activity gradually decreases during aging of the rat (Alexandre et al. 2013), which could impair the comparison between different studies. Finally, RSIE showed low trehalase and palatinose activities whose specific values were in agreement with previous work (Oku et al. 2011).

Table 1. Protein Content and Enzymatic Activities of Rat Small Intestine Extract (RSIE) Measured at the Studied Conditions.

Activity	Substrate	Condition (pH; T)	U (μmol/min g)
β-galactosidase	*o*-NPG[a]	7.0; 37 ˚C	26.7 ± 2.0[b]
Maltase	*p*-NPG[a]	6.8; 37 ˚C	443.2 ± 2.0[c]
Sucrase	Sucrose[d]	6.8; 37 ˚C	23.5 ± 0.7[c]
Palatinase	Palatinose[d]	6.8; 37 ˚C	3.3 ± 0.4[c]
Trehalase	Trehalose[d]	6.8; 37 ˚C	6.8 ± 1.0[c]

Protein content of RSIE: 8.9 ± 0.4% (w/w).
[a] Enzyme activity determined by measuring the absorbance of released NP at 420 nm.
[b] Values are expressed as means ± SD (*n*=6).
[c] Values are expressed as means ± SD (*n*=3).
[d] Enzyme activity determined by measuring the absorbance of released reducing sugars at 540 nm.

Small intestinal digestion of digestible carbohydrates

Figure 1 shows the evolution of maltose, sucrose and lactose throughout the intestinal digestion process with the extract. Although substantial hydrolysis rates were observed in all cases, dissimilar trends were observed for each carbohydrate. Thus, maltose was rapidly and fully digested as it disappeared after 15 min of digestion, which was the first sampling time (**Figure 1A**). Sucrose showed a slower but also high digestion rate, achieving a relative hydrolysis rate of 88.1% at the end of the digestion (**Figure 1B**), whereas lactose was the less hydrolysed substrate with a maximum degradation of 55.8% (**Figure 1C**). Among the human and rat dissacharidases, β-galactosidase has been reported to have the lowest activity which could explain the lower hydrolysis rate of lactose as compared to maltose and sucrose (Oku et al. 2011).

Small intestinal digestion of prebiotic carbohydrates

The digestibility of a total of six carbohydrates classified as well-known (lactulose, GOS-1, GOS-2 and FOS) or potentially prebiotics (lactosucrose and OsLu) was assessed using RSIE and, conveniently, monitored and quantified by GC-FID in order to draw insights from the partial breakdown, if any, of the tested prebiotics.

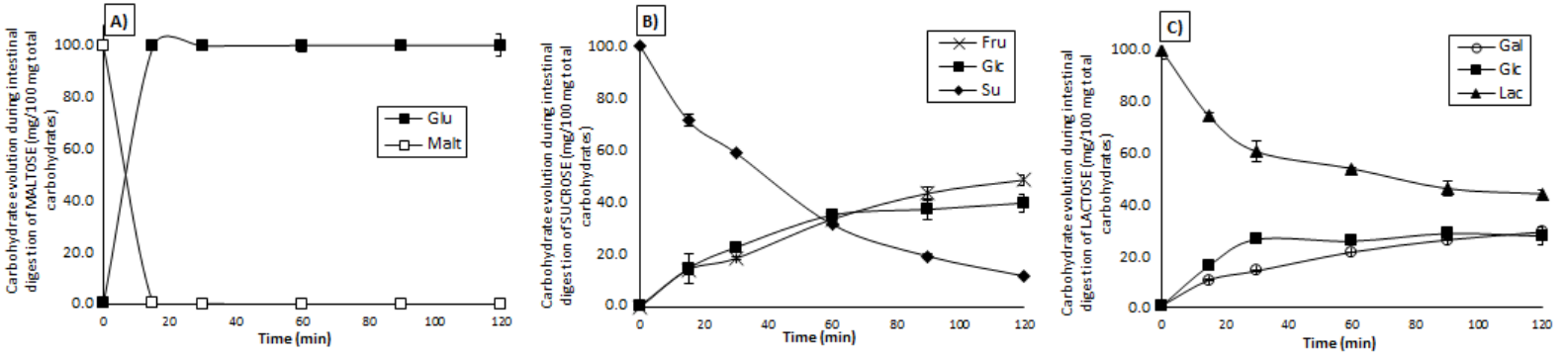

Figure 1. Hydrolysis rates of maltose (A), sucrose (B) and lactose (C) and their released monosaccharides upon small intestinal digestion at 37 ˚C, pH 6.8 for 2 hours using RSIE.

Single carbohydrates as lactosucrose and, specially, lactulose, showed a high resistance to the intestinal digestion, resulting in low hydrolysis degrees of 26.0% and 11.1% (**Table 2**), respectively, after 2 h of digestion.

Remarkably, GOS-2, with $\beta(1\rightarrow6)$ predominant linkage, showed a significantly higher overall resistance to intestinal digestion (23.3% of hydrolysis degree at the end of digestion) than GOS-1 (34.2% of hydrolysis degree), whose main linkage is $\beta(1\rightarrow4)$, highlighting the key role played by the glycosidic linkage involved in the oligosaccharide chain.

Novel galacto-oligosaccharides derived from lactulose (OsLu) presented an overall hydrolysis degree (i.e., 18%) which was significantly higher than that of lactulose only at longer digestion times (90 and 120 min), whereas no significant differences between both carbohydrates were observed during the first hour of digestion. Nevertheless, OsLu had a hydrolysis degree significantly lower than those of GOS-2 and, specially, GOS-1 throughout the digestion process (**Table 2**). OsLu and GOS-2 are mainly comprised of oligosaccharides containing $\beta(1\rightarrow6)$ as the main glycosidic linkage but they differ in the presence of fructose at the reducing end of OsLu instead of glucose. Therefore, this result reveals that the monomer composition is also a critical factor for carbohydrate digestibility. These data are in good agreement with previous findings described by Hernández-Hernández et al.

(2012), who reported a lower ileal digestibility of OsLu as compared to GOS following an *in vivo* approach using rats. In addition, also in line with our findings, these authors observed that $\beta(1\rightarrow6)$ and $\beta(1\rightarrow2)$ linkages between galactose and glucose monomers were significantly more resistant to *in vivo* gastrointestinal digestion than the $\beta(1\rightarrow4)$ linkage between galactose units within the GOS mixture. According to these comparative findings, it could be inferred that the *in vitro* digestion model developed in the present work is suitable for replacing *in vivo* rat models, stressing the usefulness of the RSIE as a reliable, simple and cost-effective tool to assess carbohydrate digestibility. Recently, OsLu have also shown to be more resistant to *in vitro* digestion than conventional GOS following their inclusion in milk (Ferreira-Lazarte et al. 2017a).

Finally, FOS, a mixture comprised of kestose and nystose, were also less prone to intestinal degradation than GOS-1 and GOS-2, showing a low hydrolysis degree of 12.0% after 2 h of digestion (**Table 2**). These data confirm the high resistance to mammalian digestive enzymes of $\beta(2\rightarrow1)$ linkages previously observed in FOS (Roberfroid et al. 2010; Oku et al. 1984). In addition, the overall hydrolysis degree obtained with the current *in vitro* digestion model is fairly similar to a previous *in vivo* study carried out with healthy humans and based on aspiration of the gut content at the

terminal ileum (Molis et al. 1966). Concretely, up to 89% of ingested FOS in a single meal was recovered in intact form. Consequently, these authors indicated that around 11% of FOS was hydrolysed by either acidic conditions or digestive enzymes in the small intestine.

Table 2. Hydrolysis Degree [A] (%) of Non-Digestible Carbohydrates during the Small Intestinal Digestion Treatment using RSIE at 37 ˚C, pH 6.8.

Digestion time (min)	Lactulose	GOS-1[B]	GOS-2[B]	OsLu[C]	FOS[D]	Lactosucrose
15	3.2 ± 0.5 [a]	13.2 ± 1.7 [c]	9.7 ± 1.5 [b]	2.5 ± 1.3 [a]	2.9 ± 0.1 [a]	7.4 ± 0.8 [b]
30	4.5 ± 0.3 [a]	21.1 ± 1.2 [d]	15.7 ± 1.5 [c]	6.2 ± 1.0 [a]	4.3 ± 0.2 [a]	10.4 ± 0.4 [b]
60	7.9 ± 0.1 [a]	30.1 ± 1.0 [c]	18.0 ± 1.1 [b]	8.1 ± 0.8 [a]	7.2 ± 0.3 [a]	18.0 ± 1.4 [b]
90	8.9 ± 0.2 [a]	34.2 ± 1.3 [d]	22.4 ± 1.7 [c]	16.0 ± 1.1 [b]	10.3 ± 0.4 [a]	21.4 ± 2.3 [c]
120	11.1 ± 0.1 [a]	34.2 ± 0.5 [d]	23.3 ± 0.7 [c]	18.0 ± 3.2 [b]	12.0 ± 0.6 [a]	26.0 ± 1.7 [c]

[A] Data are expressed as the mean ± SD (n=4).
[B] Hydrolysis degree (%) based on the joint digestibility of di-, tri- and tetrasaccharide fractions.
[C] Hydrolysis degree (%) based on the joint digestibility of di- and trisaccharide fractions.
[D] Hydrolysis degree (%) based on the joint digestibility of kestose and nystose.
[a, b, c] Different letters indicate statistical differences between all tested carbohydrate samples at the same reaction time using a one-way analysis of variance (ANOVA) ($p < 0.05$) (n=4).

Table 3 shows the individual content in the monosaccharide fraction of all prebiotic oligosaccharides assayed, as well as the joint content of di-, tri- and tetrasaccharide fractions in GOS and OsLu mixtures. In agreement with the overall hydrolysis degree displayed in **Table 2**, in GOS-1 the minor tetrasaccharide fraction was substantially reduced and also, although at a lesser extent, the tri- and disaccharide fractions, whereas GOS-2 and OsLu presented substantial hydrolysis only in the disaccharide fraction (**Table 3**). Furthermore, **Figures 2** and **3** illustrate the different resistance of the

individual carbohydrates of GOS-1 and OsLu, respectively, to intestinal digestion based on their corresponding GC-FID profiles. Peaks 5 and 7, identified as 4'-galactosyl-lactose (*β*-D-Gal(1→4)-*β*-D-Gal-(1→4)-D-Glc) and 4'-digalactosyl-lactose (*β*-D-Gal(1→4)-*β*-D-Gal(1→4)-*β*-D-Gal-(1→4)-D-Glc), respectively (Cardelle-Cobas et al. 2009b), were the main tri- and tetrasaccharide present in GOS-1 and clearly diminished after two hours of RSIE digestion (**Figure 2**). However, peak 4, identified as allolactose (*β*-D-Gal-(1→6)-D-Glc), an isomer of lactose having a *β*(1→6) linkage, and the minor peak 8, identified as 6'-digalactosyl-lactose (*β*-D-Gal(1→6)-*β*-D-Gal(1→6)-*β*-D-Gal-(1→4)-D-Glc), appeared to be fully resistant to RSIE digestion. In contrast, no measurable differences were observed in any of the individual chromatographic peaks corresponding to the trisaccharide fractions of OsLu, and only very small decreases could be detected in the disaccharide fraction (**Figure 3**). Therefore, the contribution to the overall hydrolysis degree of OsLu displayed in **Table 2** seems to be due basically to the disaccharide fraction (**Table 3**).

Table 3. Carbohydrate Content Determined by GC-FID Analysis in Non-Digestible Oligosaccharides during the Small Intestinal Digestion Treatment using RSIE at 37 ˚C, pH 6.8.

Sample	Reaction time	Carbohydrate content (mg/100 mg of total carbohydrates)[a]								
		Fructose	**Galactose**	**Glucose**	**Lactose**	**Lactulose**	**Disaccharides**	**Trisaccharides**	**Tetrasaccharides**	**Total OS[b]**
Lactulose	blank	0.0 ± 0.0	0.0 ± 0.0	N.D.	N.D.	100.0 ± 0.0	N.D.	N.D.	N.D.	N.D.
	15 min	1.1 ± 0.1	2.1 ± 0.4	N.D.	N.D.	96.8 ± 0.5	N.D.	N.D.	N.D.	N.D.
	30 min	2.1 ± 0.2	2.4 ± 0.2	N.D.	N.D.	95.5 ± 0.3	N.D.	N.D.	N.D.	N.D.
	60 min	3.6 ± 0.1	4.3 ± 0.1	N.D.	N.D.	92.1 ± 0.1	N.D.	N.D.	N.D.	N.D.
	90 min	4.3 ± 0.1	4.4 ± 0.4	N.D.	N.D.	91.3 ± 0.5	N.D.	N.D.	N.D.	N.D.
	120 min	5.1 ± 0.1	6.1 ± 0.1	N.D.	N.D.	88.8 ± 0.1	N.D.	N.D.	N.D.	N.D.
GOS-1	blank	N.D.	1.2 ± 0.0	27.1 ± 0.5	16.5 ± 0.3	N.D.	24.3 ± 0.4	26.3 ± 0.4	4.6 ± 0.6	55.2 ± 0.7
	15 min	N.D.	7.9 ± 0.2	29.3 ± 1.0	14.8 ± 0.4	N.D.	20.7 ± 0.4	24.1 ± 0.3	3.1 ± 0.3	48.0 ± 1.0
	30 min	N.D.	12.5 ± 0.8	29.8 ± 0.7	14.1 ± 0.4	N.D.	20.0 ± 0.3	21.5 ± 0.5	2.1 ± 0.2	43.6 ± 0.9
	60 min	N.D.	16.6 ± 0.6	32.4 ± 1.0	12.4 ± 0.3	N.D.	17.2 ± 0.3	19.7 ± 0.3	1.7 ± 0.4	38.6 ± 0.9
	90 min	N.D.	22.2 ± 1.0	30.1 ± 1.4	11.3 ± 0.2	N.D.	17.1 ± 0.6	17.7 ± 0.2	1.4 ± 0.1	36.3 ± 0.8
	120 min	N.D.	23.7 ± 0.9	30.1 ± 0.8	10.6 ± 0.2	N.D.	16.7 ± 0.3	17.7 ± 0.4	1.7 ± 0.1	36.3 ± 0.3
GOS-2	blank	N.D.	9.2 ± 0.1	31.0 ± 0.1	26.0 ± 0.1	N.D.	13.5 ± 0.1	19.4 ± 0.1	0.9 ± 0.1	33.8 ± 0.1
	15 min	N.D.	14.0 ± 0.5	33.3 ± 0.6	22.1 ± 0.7	N.D.	11.5 ± 0.1	18.4 ± 0.6	0.6 ± 0.1	30.5 ± 0.6
	30 min	N.D.	16.3 ± 1.0	36.5 ± 1.5	18.7 ± 0.2	N.D.	10.6 ± 0.5	17.5 ± 1.6	0.4 ± 0.1	28.5 ± 1.2
	60 min	N.D.	18.4 ± 0.4	36.5 ± 1.3	17.3 ± 0.5	N.D.	9.4 ± 0.4	17.8 ± 0.3	0.6 ± 0.1	27.7 ± 0.5
	90 min	N.D.	18.1 ± 0.5	41.7 ± 0.1	13.9 ± 0.3	N.D.	7.9 ± 0.3	17.3 ± 0.4	1.0 ± 0.1	26.3 ± 0.6
	120 min	N.D.	20.2 ± 0.1	40.8 ± 1.8	12.9 ± 0.2	N.D.	7.9 ± 0.4	17.1 ± 1.2	0.8 ± 0.1	25.9 ± 1.5
OsLu	blank	0.6 ± 0.0	16.7 ± 0.3	N.D.	N.D.	34.7 ± 0.1	26.2 ± 0.1	21.3 ± 0.2	N.D.	47.5 ± 0.2
	15 min	0.9 ± 0.3	20.8 ± 0.9	N.D.	N.D.	31.9 ± 0.9	23.5 ± 0.8	22.8 ± 0.8	N.D.	46.5 ± 0.8
	30 min	0.9 ± 0.2	23.0 ± 0.8	N.D.	N.D.	31.4 ± 0.7	22.6 ± 0.6	22.0 ± 1.0	N.D.	44.6 ± 0.5
	60 min	1.2 ± 0.1	24.0 ± 0.7	N.D.	N.D.	31.1 ± 0.4	20.0 ± 0.3	23.7 ± 0.4	N.D.	43.7 ± 0.4
	90 min	2.2 ± 0.1	27.6 ± 0.8	N.D.	N.D.	30.2 ± 0.1	18.9 ± 0.5	21.1 ± 1.0	N.D.	39.9 ± 0.5
	120 min	2.0 ± 0.2	28.1 ± 1.4	N.D.	N.D.	30.6 ± 0.4	17.8 ± 0.1	21.0 ± 1.9	N.D.	38.9 ± 1.8
		Fructose	**Galactose**	**Glucose**	-	**Sucrose**	-	**Kestose**	**Nystose**	**FOS**
FOS	blank	0.3 ± 0.0	N.D.	N.D.		2.7 ± 0.1		65.2 ± 1.1	31.8 ± 1.2	97.0 ± 0.1
	15 min	2.5 ± 0.1	N.D.	N.D.		3.3 ± 0.2		65.1 ± 0.8	29.1 ± 0.8	94.2 ± 0.1
	30 min	3.8 ± 0.1	N.D.	N.D.		3.3 ± 0.2		65.4 ± 1.0	27.5 ± 1.2	92.8 ± 0.2
	60 min	6.6 ± 0.2	N.D.	N.D.		3.4 ± 0.1		66.6 ± 0.6	23.4 ± 0.5	90.0 ± 0.1
	90 min	9.8 ± 0.4	N.D.	N.D.		3.2 ± 0.2		67.0 ± 0.8	20.0 ± 0.9	87.0 ± 0.3
	120 min	11.6 ± 0.4	N.D.	N.D.		3.1 ± 0.2		67.4 ± 0.5	18.0 ± 0.1	85.4 ± 0.5
Lactosucrose		**Fructose**	**Galactose**	**Glucose**	**Lactose**	**Sucrose**	-	**Lactosucrose**	-	
	blank	0.1 ± 0.1	0.0 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.0		98.8 ± 0.3		
	15 min	0.8 ± 0.1	0.6 ± 0.1	2.5 ± 0.4	1.6 ± 0.1	3.0 ± 0.1		91.5 ± 0.6		
	30 min	1.4 ± 0.1	1.5 ± 0.1	2.7 ± 0.4	2.1 ± 0.1	3.8 ± 0.1		88.5 ± 0.4		
	60 min	2.5 ± 0.2	2.9 ± 0.2	5.9 ± 0.9	3.3 ± 0.2	4.6 ± 0.1		81.0 ± 1.4		
	90 min	3.9 ± 0.1	4.2 ± 0.2	4.5 ± 2.3	4.6 ± 0.1	5.2 ± 0.1		77.7 ± 2.3		
	120 min	5.4 ± 0.3	5.5 ± 0.4	4.9 ± 0.9	5.9 ± 0.5	5.3 ± 0.2		73.1 ± 0.7		

[a] Data are expressed as the mean ± SD (n=4).
[b] Total oligosaccharide contents based on the sum of di-, tri- and tetrasaccharide fractions.

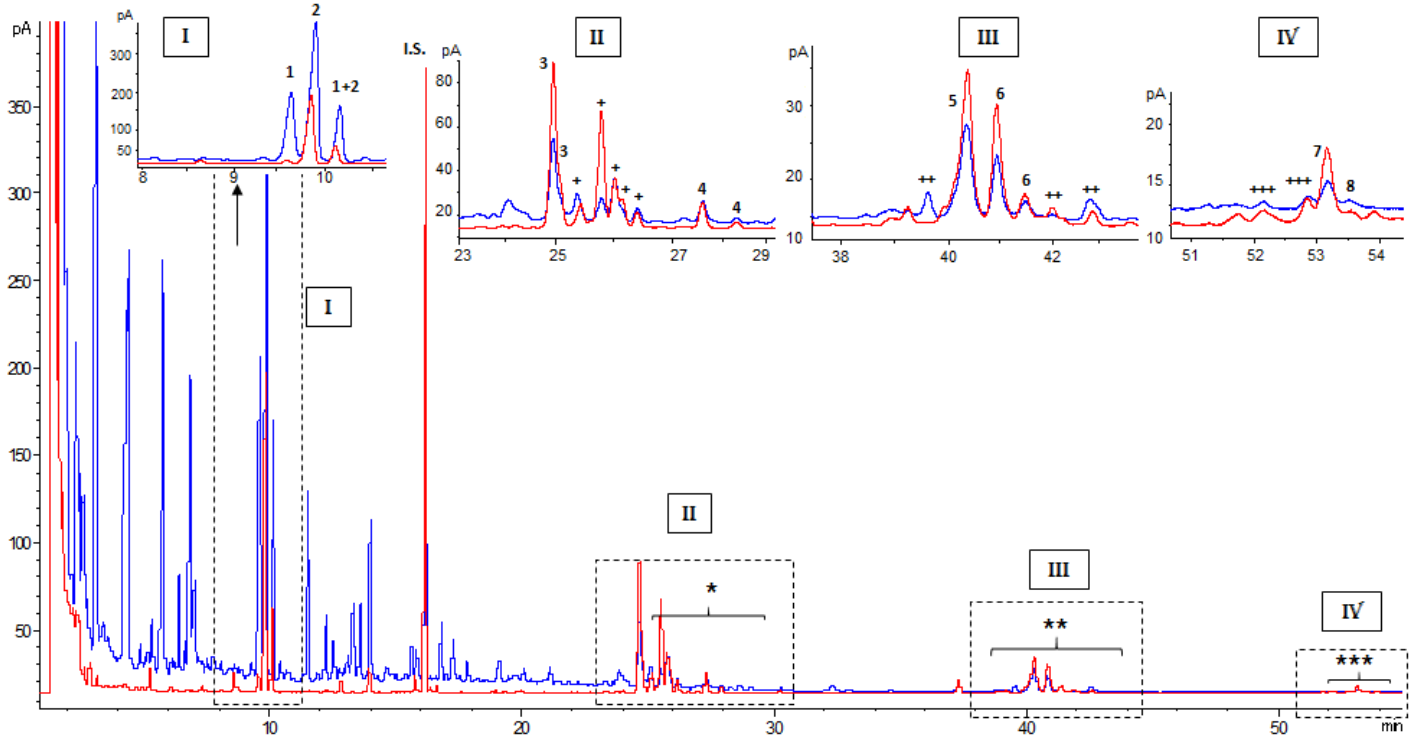

Figure 2. GC-FID profiles of TMSO derivatives of carbohydrates present in GOS-1 undigested (red) and after 2 h of small intestinal digestion with RSIE (blue). Peaks: 1: Galactose, 2: Glucose, I.S.: Internal standard (phenyl-β-D-glucoside), 3: Lactose, 4: Allolactose, 5: 4'-galactosyl-lactose, 6: 6'-galactosyl-lactose + unknown peak, 7: 4'-digalactosyl-lactose, 8: 6'-digalactosyl-lactose.
+ Other disaccharides, ++ other trisaccharides, +++ other tetrasaccharides. * Disaccharides were considered the sum of peaks 3 to 4. ** Trisaccharides were the sum of peaks 5, 6 and other trisaccharides. *** Tetrasaccharides were quantified as the sum of peaks 7, 8 and other tetrasaccharides.

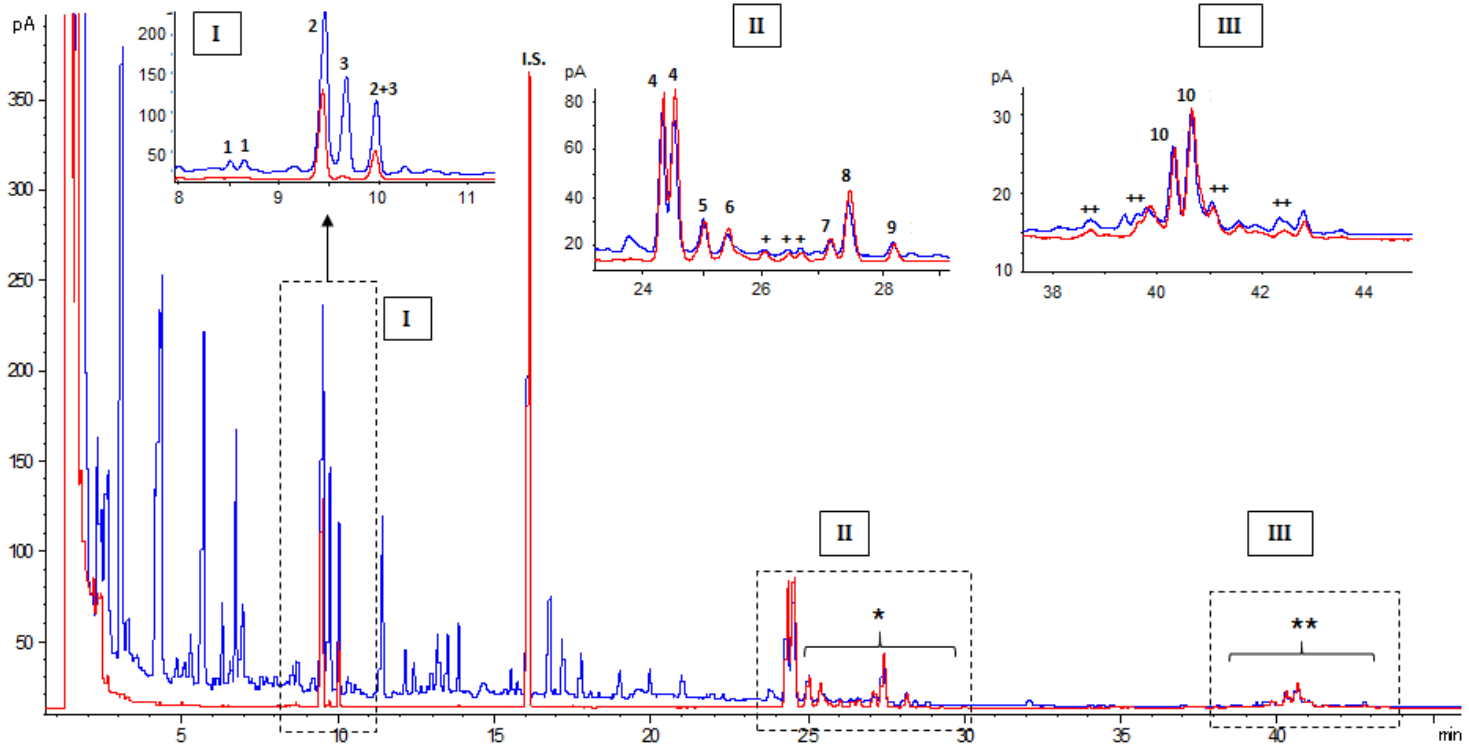

Figure 3. GC-FID profiles of TMSO derivatives of carbohydrates present in OsLu undigested (red) and after 2 h of small intestinal digestion with RSIE (blue). Peaks: 1: Fructose, 2: Galactose, 3: Glucose, I.S.: Internal standard (phenyl-β-D-glucoside), 4: Lactulose, 5: 1,1-galactobiose, 6: 1,3-galactobiose, 7: 1,1-galactosyl-fructose 1, 8: 1,6-galactobiose E, 9: 1,6-galactobiose Z, 10: 6'-galactosyl-lactulose.
+ Other disaccharides, ++ Other trisaccharides. * Disaccharides were considered the sum of peaks 5 to 9. ** Trisaccharides were the sum of peaks 10 and other trisaccharides.

Regarding FOS, nystose was partially hydrolysed and, probably, converted to kestose as indicated by the slight increase found in fructose content and the non-detection of released glucose and/or the trisaccharide inulotriose (*β*-D-Fru(2→1)-*β*-D-Fru(2→1)-*β*-D-Fru) (**Table 3**). This could be indicative of a higher lability of the linkage *β*(2→1) when is bonding fructose monomers instead of fructose and glucose in inulin-type FOS. Finally, the partial hydrolysis of lactosucrose gave rise to the release of similar levels of sucrose and lactose indicating, thus, no particular preference of the digestive enzymes between the *β*(1→4) linkage of the lactose moiety and the *β*(2→1) linkage of the sucrose moiety.

To sum up, nine dietary carbohydrates, three digestible and six considered as non-digestible, were subjected to digestion using RSIE combined with physiological conditions (i.e., temperature and pH) and their hydrolysis products were comprehensively analysed and quantified by GC-FID. The results confirmed the high and readily digestibility of maltose and sucrose, followed distantly by lactose. In any case, either the well-known or the potential prebiotics showed a higher resistance to RSIE digestion although partial hydrolysis at different extent was observed in all tested carbohydrates. Thus, FOS (a mixture of kestose and nystose) and lactulose were the most resistant carbohydrates to intestinal digestion, followed closely

by OsLu and, then, by GOS-2, lactosucrose and, finally, GOS-1 (**Tables 2** and **3**). To the best of our knowledge, the present data are the first comparing the digestibility rates of two types of GOS differing in the predominant glycosidic linkage, revealing the higher resistance of $\beta(1\rightarrow6)$ than $\beta(1\rightarrow4)$ linkages to rat digestive enzymes. Moreover, the observed differences between OsLu and GOS-2 also pointed out the role of the monomer composition and, more concretely, the higher resistance of galactosyl-fructoses than galactosyl-glucoses.

There are currently very few studies and reliable data on the digestibility of potentially non-digestible carbohydrates, despite their increasingly important role in human health. The *in vitro* digestion model, based on the use of RSIE under physiological conditions of temperature and pH, described in this work has shown to be a useful, simple and cost-effective tool to evaluate the digestibility of dietary oligosaccharides. In general terms, the described method allows the distinction between digestible and non-digestible carbohydrates of degree of polymerization up to four, as the tested digestible carbohydrates were readily hydrolysed whereas the oligosaccharides classified as non-digestible were barely or significantly less hydrolysed than the digestible carbohydrates. The combination of RSIE digestion with sensitive and powerful separation methods, such as GC-FID, instead of

colorimetric methods as it has been traditionally performed, allows much more informative read-outs of the digestion process (e.g., lability of different glycosidic linkages, determination of the released carbohydrates resisting digestion). In addition, the developed *in vitro* digestion model has the advantage of requiring a minimum quantity of carbohydrates (0.5 mg), which is typically a limiting factor when the digestibility of novel carbohydrates produced at laboratory scale is assessed.

Chapter 2

Study on the digestion of milk with prebiotic carbohydrates in a simulated gastrointestinal model

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Abstract:

The behaviour of oligosaccharides from lactulose (OsLu) included with milk was examined during in vitro gastrointestinal digestion using the Infogest protocol as well as some small intestine rat extract. The digestion was compared with commercial prebiotics GOS and Duphalac®. Electrophoretic analysis demonstrated that the prebiotic carbohydrates did not modify the gastric digestion of dairy proteins. Similarly, no significant effect of gastrointestinal digestion was shown on the prebiotic studied. In contrast, under the intestinal conditions using a rat extract, the oligosaccharides present in OsLu samples were less digested (<15%) than in GOS (35%). Moreover, lactulose was more prone to digestion than their corresponding trisaccharides. These results demonstrate the limited digestion of OsLu and their availability to reach the large intestine as prebiotic.

Introduction:

Prebiotics can reach the distal portions of the colon to selectively stimulate the growth of bifidobacteria and lactobacilli, providing important benefits to health (Gibson et al. 2004). The most relevant compounds are oligosaccharides. These prebiotics may exert other bioactive properties such as improving mineral absorption and metabolic disorders and slow gastric emptying, among other effects (Moreno et al. 2014).

Several commercial preparations of galactooligosaccharides (GOS) and fructooligosaccharides (FOS) are used as prebiotic ingredients in some foods such as infant formula and dairy products (Sabater et al. 2016). Lactulose (i.e. lactose isomer) is also a recognized prebiotic for the treatment of constipation and systemic portal encephalopathy (Schumann et al. 2002; Olano & Corzo 2009). Given the huge interest in recent years towards the gastrointestinal function and new structures with improved properties, new routes to obtain a second-generation of prebiotic oligosaccharides are being explored (Moreno et al. 2017). This is the case of the oligosaccharides derived from lactulose (OsLu). These prebiotic mixtures, obtained by enzymatic synthesis using β-galactosidases from microbial origin, might impart better prebiotic properties than commercial GOS (Moreno et al. 2014).

One of the requirements for oligosaccharides to be considered as prebiotics is their resistance to digestion in the upper gastrointestinal tract.

The susceptibility of prebiotic oligosaccharides to hydrolysis during their passage through the gastrointestinal tract is largely affected by the chemical structure and can impact their final state when they reach the colon to be fermented by the microbiota. Ohtsuka et al. (1990) found that the trisaccharide 4'-galactosyl-lactose was hardly digested *in vitro* with a homogenate of intestinal mucosa of rats. According to Torres et al. (2010), more than 90% of GOS are stable to digestive enzymes and can reach the colon to exert their positive effect. Carbohydrate analysis before and after exposure to certain protocols of *in vitro* digestion have shown that xylo-oligosaccharides, palatinose condensates, commercial GOS and lactulose were very resistant to hydrolysis, In contrast, lactosucrose, gentio-oligosaccharides, soybean oligosaccharides, fructo-oligosaccharide and inulin were slightly hydrolysed under such conditions (Playne and Crittenden 2009).

To our knowledge, limited studies have been carried out on the digestibility of OsLu. Hernandez-Hernandez et al. (2012) pointed out in *in vivo* assays a higher resistance of OsLu compared to GOS during gastrointestinal digestion. This was ascribed to the presence of fructose in $\beta(1\rightarrow4)$ linkage with galactose at the reducing end of the OsLu molecules. However, there is a lack of studies on the susceptibility of OsLu to the gastrointestinal digestion when they are added in a food matrix and the

impact of these compounds on the digestion of other food components. These considerations are important since standards would be more prone to changes as they are not protected in a food medium. Establishing the digestibility of prebiotic carbohydrates is of great practical application, since this influences on the final dose of substrate that reaches the distal portions of gut to exert its prebiotic effect. Thus, the aim of this work has been to study the effect of the OsLu inclusion in milk on the digestion of proteins and the changes in the carbohydrate fraction using standardised *in vitro* digestive conditions with a more physiological relevant gastric digestion approach. A subsequent treatment with a rat small intestine extract has been included to study the effect of intestinal enzymes from mammals. The commercial prebiotics GOS and Duphalac® were also employed for comparison purposes.

Materials and methods:

Chemicals and reagents. Galactose, D-glucose, fructose, lactose, lactulose, raffinose, stachyose, phenyl-β-glucoside and intestinal acetone powders from rat (rat intestine extract, RSIE) from Sigma-Aldrich chemical Company (St Louis, MO).

Obtainment of prebiotic ingredients. OsLu were obtained at pilot scale by Innaves S.A. (Vigo, Spain) following the method described by Anadón et al. (2013). In brief, OsLu were synthesised using a commercial lactulose

preparation (670 g/L; Duphalac®, Abbott Biologicals B.V., Olst, The Netherlands), diluted with water to 350 g/L and pH adjusted to 6.7 with KOH, and *β*-galactosidase from *Aspergillus oryzae* (16 U/mL; Sigma), selected by its high yield for synthesis of OsLu (Cardelle-Cobas et al. 2016). Enzymatic reactions were carried out at 50 ˚C in an orbital shaker at 300 rpm for 24 h. Afterwards, samples were immediately immersed in boiling water for 10 min to inactivate the enzyme. The mixture of oligosaccharides (20% [w/v]) was treated with fresh *Saccharomyces cerevisiae* (1.5% [w/v]; Levital, Paniberica de Levadura S.A., Valladolid, Spain) at 30ºC and aeration at 20 L/min, to decrease the monosaccharides content (Sanz et al. 2005). Finally, the samples were vacuum concentrated at 40 ºC in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). GOS syrup was kindly provided by Friesland Campina Domo (Hanzeplein, The Netherlands).

Milk samples. Skim Milk Powder (low-heat organic, protein 42.34%, fat 0.89%, lactose 49.8% (w/w) (SMP) was kindly provided by Fonterra NZ. The SMP was reconstituted at 10% with distilled water and, subsequently, lactulose (Duphalac®), GOS or OsLu were added at 5% (w/w), taking into account previous recommendations for prebiotic doses (3.3 g of prebiotic carbohydrates/100 mL) (Walton et al. 2012; Whisner et al. 2013; López-Sanz et al. 2015). The samples were labeled as SMP+Duphalac®, SMP+GOS and SMP+OsLu and were kept refrigerated until subsequent assays.

***In vitro* gastrointestinal digestion.** The solutions (see **Figure 1**) used for the simulation of the oral and gastric phases were based on the standardized static digestion protocol Infogest (Minekus et al. 2014). 5 mL of sample was placed into a 70 mL glass v-form vessel thermostated at 37 ºC. To simulate the oral phase, 4 mL of Simulated Salivary Fluid (SSF, (Table 1S, *Annex A*) Verhoeckx et al. (2015), 25 µL 0.3 M $CaCl_2(H_2O)$ and 0.975 mL Milli-Q water were added and mixed for approximately 2 min using a 3D action shaker (Mini-gyro rocker-SSM3-Stuart, Barloworld Scientific limited, UK) at 35 rpm. The simulation of the gastric phase was conducted using a semi-dynamic model described by Mulet-Cabero et al. (2017). The gastric fluids and enzyme solution were added gradually. Two solutions were added at a constant rate for 2 h: (1) 9 mL of a mixture consisted of 88.9% Simulated Gastric Fluid (SGF), 0.06% 0.3 M $CaCl_2(H_2O)$, 4.4% Milli-Q water and 6.7% 2 M HCl was added using the dosing device of an autotitrator (836 Titrando-Metrohm, Switzerland) and (2) 1 mL of pepsin (3,214 U/mg solid, using haemoglobin as substrate) solution (in water) was added to reach the protease activity of 2,000 U/mL in the final digestion mixture. This enzyme solution was added using a syringe pump (Harvard apparatus, PHD ultra, USA). The system was agitated using the 3D action shaker at 35 rpm during the digestion time.

The pH was recorded throughout the procedure. Samples (0.5 mL) were taken after 0, 1 and 2 h of digestion and the pepsin activity was stopped with 100 µL of 1 M $NaHCO_3$ for a subsequent analysis of the protein fraction and the rest of the sample with 150 µL of 5 M NaOH for the following intestinal digestion. This last sample was labelled as GPhase sample. After gastric digestion two different procedures for small intestinal digestion were carried out:

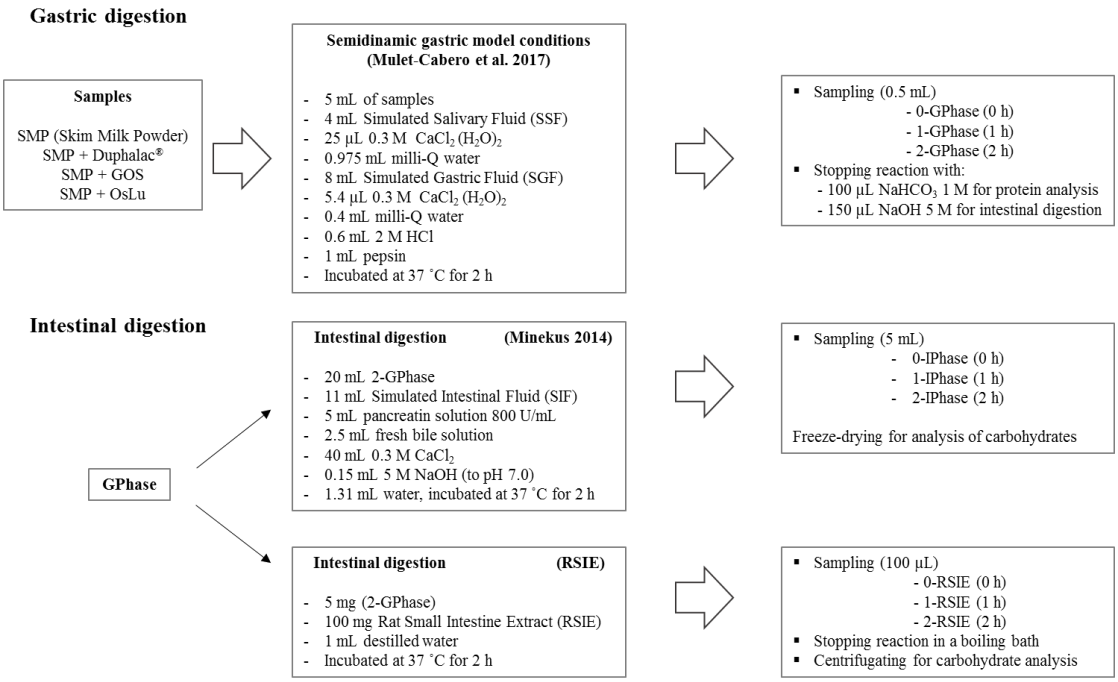

Figure 1. Scheme of the experimental procedure.

i) 2 mL of GPhase was freeze-dried and kept at -20°C until used for intestinal digestion assays with a crude enzyme of rat small intestine extract (RSIE). 5 mg of GPhase was mixed with 100 mg of RSIE and 1 mL distilled water. The mixture was incubated at 37° for 2 h, taking samples after 0, 0.5, 1 and 2 h. These samples were centrifuged at 10,000 rpm for 2 min and 100 µL of the supernatant was taken for carbohydrate analysis.

ii) The rest of the liquid GPhase (~ 16.5 mL) was subjected to the small intestine conditions following the Infogest Protocol (Minekus et al., 2014). The digestion was carried out at 37°C for 2 h. Samples (5 mL) were taken at 0, 1 and 2 h of small intestinal digestion, which were respectively labelled as 0-IPhase, 1-IPhase and 2-IPhase. They were freeze-dried until further analysis.

Protein determination. The changes in the protein fraction during gastric digestion of milk containing prebiotic ingredients (GPhase 0, 1 and 2 h) were followed by SDS-PAGE. 65 µL of sample was mixed with 25 µL of 4X NuPAGE LSD sample buffer (Invitrogen, Carlsbad, California, USA) and 10 µL of 8% dithiothreitol. The mixture was heated at 70 ºC for 10 min. 20 µL of mixture was loaded on a 12% polyacrylamide NuPAGE Novex Bis-Tris precast gel (Invitrogen,Carlsbad, California, USA) and RunBlue Precast SDS-PAGE gel cassette (Expedeon Ltd., Cambridgeshire, United Kingdom).

SDS-PAGE was performed according to the manufacture's instructions. Mark 12 Unstained Standard (Invitrogen) was used as a molecular weight marker (ranging from 2.5 to 200 kDa).

Carbohydrate analysis by GC-FID. Trimethyl silylated oximes (TMSO) of carbohydrates (mono-, di- and trisaccharides) present in samples were determined by Gas Chromatography following the method described by Montilla et al. (2009). Samples corresponding to 0.5 mg of saccharides were added to 0.2 mL of Internal Standard (I.S.) solution which contained 0.5 mg/mL of phenyl-*β*-glucoside. Response factors respect to I.S. were calculated after the duplicate analysis of standard solutions (fructose, galactose, glucose, lactose, lactulose, sucrose, raffinose and stachyose), at different concentrations ranging from 0.005 to 4 mg/mL.

Statistical analysis. All digestions were carried out in duplicate and analyses were also performed in duplicate (n=4). The comparison of means was carried out using one-way analysis of variance (Tukey HSD Multiple Range Test). Statistical analyses were performed using the SPSS statistical package (Inc., Chicago, Il). The differences were considered significant when $p < 0.05$.

Results and discussion:

Effect on protein digestion. Figure 1S (*Annex A*) shows the pH profile of the different samples of SMP with the addition of prebiotic ingredients (Table 2S, *Annex A*, carbohydrate composition analysed by GC-FID) during their digestion in the semi-dynamic gastric model. The initial pH values were close to 7 in all cases and gradually decreased to 1.8 at the end of the gastric digestion. In general, the profiles of the milk samples with prebiotic ingredients were similar to that of the SMP (no prebiotic ingredient added). The gradual lowering of pH enables the restructuring of the proteins due to acid induced coagulation to be simulated and is based on typical pH profiles measured *in vivo* (Malagelada et al. 1979).

The electrophoretic profile of proteins corresponding to samples 0, 1 and 2 h of gastric digestion are illustrated in **Figures 2** and **3**. These figures show bands of pepsin, caseins, BSA, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). In the case of mixtures with OsLu and GOS at 0 h (**Figure 2**) more intense bands appeared in the area corresponding to α-La, probably due to the formation of complexes between the protein and carbohydrates, which disappeared during the digestion. In general, after 2 h of gastric digestion, the bands corresponding to undigested proteins from both SMP and SMP with added prebiotics were not detected with the exception of β-Lg which has

been shown to be more resistant to pepsin hydrolysis (Mandalari et al. 2009). **Figure 3** shows some diffuse, low molecular weight bands in samples corresponding to 1 and 2 h of digestion which could be related to small molecular weight peptides formed after milk protein digestion (lanes 5-12). The intensity of these bands was estimated by the Quantity One software. This showed an increase of intensity with digestion time obtaining values of 0.54 at 0.62 after 1 h and 0.64 at 0.75 after 2 h, with the lowest values corresponding to skim milk control.

These results show that the SDS-PAGE profile of milk with prebiotic carbohydrates was similar to that of milk without addition of these ingredients, indicating that the presence of these prebiotics in milk at the concentration required to achieve a prebiotic effect, did not modify the gastric digestion of dairy proteins.

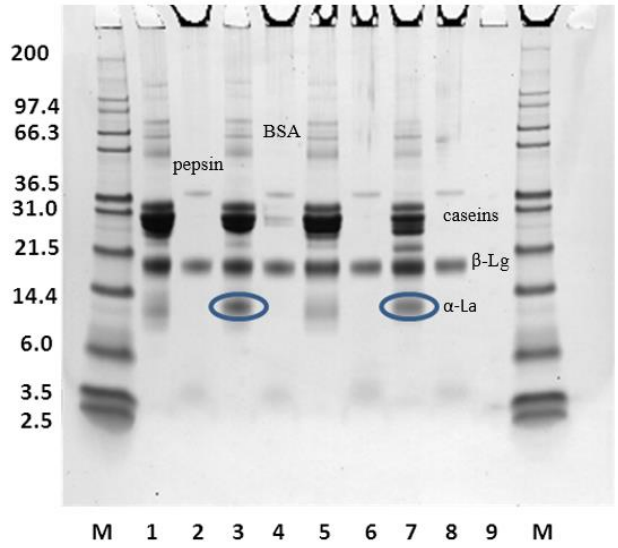

Figure 2. Electrophoretic profiles of milk protein fractions (caseins, β-Lg, α-La, BSA) before and after 2 h of digestion (Bis-Tris-Gel, Novex, NuPage). M: Marker, 1: SMP 0 h, 2: SMP 2 h, 3: SMP+OsLu 0 h, 4: SMP+OsLu 2 h, 5: SMP+ Duphalac 0 h, 6: SMP+Duphalac® 2 h, 7: SMP+GOS 0 h, 8: SMP + GOS 2 h, 9: blank

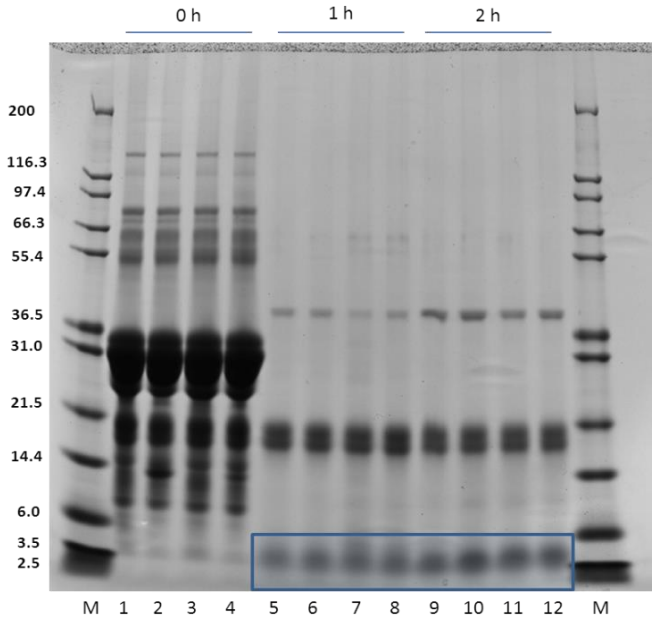

Figure 3. Electrophoretic profiles of milk protein fractions (caseins, β-Lg, α-La, BSA) during 0, 1 and 2 h of digestion (RunBlue Precast gels). M: Marker; 1, 5 and 9 SMP; 2, 6 and 10 SMP+OsLu; 3, 7 and 11 SMP+GOS; 4, 8 and 12 SMP+Duphalac. *Optical density was measured in the maximum of the peak with the Software Quantity One.

Effect on carbohydrate fraction. The effect of gastrointestinal digestion on the three different prebiotics, Duphalac®, GOS and OsLu included in milk was investigated. For this purpose, the samples from the semi-dynamic gastric model were subjected to two different intestinal digestion protocols, as indicated above (Infogest protocol or RSIE). In the case of the Infogest method, Figure 2S (*Annex A*) illustrates, as an example, the chromatogram obtained by GC-FID of TMSO derivatives of carbohydrates present in the milk samples with OsLu after gastric digestion and the beginning of the intestinal phase (G+I 0 h). The peaks corresponding to carbohydrates with degree of polymerization (DP) from 1 to 4 were found; among them galactose, lactulose and di-, tri- and tetrasaccharides derived from OsLu ingredient, and galactose, glucose and lactose from milk. Galactose was present in SMP with OsLu in higher proportion than in SMP with GOS (**Table 1**) in which the most abundant monosaccharide was glucose, due to their presence in the original prebiotic mixtures. In this respect, the addition of OsLu to milk or other products could be more interesting since OsLu presents lower proportion of caloric carbohydrates with lower glycaemic index than GOS (López-Sanz et al. 2015). As observed in **Table 1**, SMP+Duphalac® had higher concentration of lactulose than SMP+OsLu because lactulose is used as substrate during its enzymatic hydrolysis and transgalactosylation.

Table 1 – Carbohydrate evolution of milk samples during Intestinal digestion (G+I Phase), according to Infogest Protocol.

		Carbohydrate content (%)							
		Galactose	Glucose	Lactulose	Lactose	Other Disaccharides	Trisaccharides	Tetrasaccharides	Oligosaccharides*
SMP	0h	0.3 ± 0.1	0.4 ± 0.2	N.D.	99.4 ± 0.2	N.D.	N.D.	N.D.	N.D.
	1h	0.3 ± 0.1	0.5 ± 0.1	N.D.	99.2 ± 0.1	N.D.	N.D.	N.D.	N.D.
	2h	0.3 ± 0.0	0.4 ± 0.2	N.D.	99.4 ± 0.2	N.D.	N.D.	N.D.	N.D.
SMP + GOS	0h	0.5 ± 0.1	7.6 ± 1.0	N.D.	65.6 ± 3.7	11.0 ± 0.8	12.9 ± 1.8	2.4 ± 0.6	26.4 ± 3.1
	1h	0.5 ± 0.0	7.7 ± 1.5	N.D.	66.3 ± 3.3	12.0 ± 2.2	12.3 ± 1.4	3.3 ± 0.7	27.6 ± 4.2
	2h	0.5 ± 0.0	6.9 ± 0.2	N.D.	68.4 ± 1.4	10.8 ± 1.3	10.9 ± 0.7	2.4 ± 1.7	24.1 ± 1.5
SMP + Duphalac®	0h	3.6 ± 0.4	0.4 ± 0.4	22.0 ± 5,1	73.6 ± 4.9	N.D.	N.D.	N.D.	N.D.
	1h	3.4 ± 0.8	0.2 ± 0.2	20.6 ± 1,1	76.5 ± 1.1	N.D.	N.D.	N.D.	N.D.
	2h	3.1 ± 0.2	0.4 ± 0.2	21.6 ± 1,9	75.6 ± 1.7	N.D.	N.D.	N.D.	N.D.
SMP + OsLu	0h	5.0 ± 0.3	0.3 ± 0.1	6.3 ± 2.1	68.4 ± 1.7	9.8 ± 0.3	9.3 ± 0.2	0.9 ± 0.2	20.1 ± 0.6
	1h	5.0 ± 0.1	0.4 ± 0.2	7.1 ± 1.4	67.4 ± 1.3	9.8 ± 0.4	9.5 ± 0.4	0.8 ± 0.3	20.1 ± 0.3
	2h	5.3 ± 0.3	0.3 ± 0.0	6.0 ± 0.4	69.0 ± 1.1	10.2 ± 0.5	8.6 ± 1.0	0.8 ± 0.6	19.6 ± 1.6

The data are expressed as the mean ± SD (p>0.05). No statistical difference was determinates between 0, 1 and 2 h samples in all compounds using a one-way analysis of variance (ANOVA) (n=4). N.D. No detected.
*Oligosaccharides: Values represent the sum of di-, tri- and tetrasaccharides.

Limited modifications were observed in the carbohydrate fraction following digestion using the Infogest protocol. In spite of the fact that there was a slight decrease of OS and trisaccharides in SMP+GOS after 2 h of digestion, these differences were not statistically significant. None of the carbohydrates derived from the prebiotic ingredients provided any significant change, indicating their stability during this enzymatic digestion by pancreatic fluids and bile salts. Moreover, it seems to be clear that the presence of other milk components did not impact the passage of GOS, Duphalac® and OsLu throughout the gastrointestinal digestion evaluated by the Infogest protocol.

In order to gain more insight in this subject and given that the Infogest protocol is mainly focus on the digestion of proteins, this study was completed with the evaluation of carbohydrate fraction of SMP with the three prebiotic ingredients after a subsequent digestion by means of an intestinal extract of from rats, labelled as RSIE, as indicated in Materials and Methods section. **Figure 4 A, B, C, D** illustrates the evolution of each carbohydrate fraction in the SMP added with Duphalac®, GOS and OsLu after their gastric and intestinal (Infogest) and with RSIE (0.5, 1 and 2 h) of digestion. Data are expressed as percentage of hydrolysis, for lactose, lactulose and oligosaccharides, and increase of monosaccharides, taking into account the control samples immediately taken after the addition of RSIE. The hydrolysis of compounds with DP $\geq$ 2 and mainly lactose increased with time of

reaction, probably due to the presence of lactase (β-galactosidase) in the RSIE, in good agreement with the increase of the monosaccharide proportion.

In general, lactose was more hydrolysed than lactulose due to the presence of fructose instead of glucose in the β linkage of the latter (Olano & Corzo, 2009), being SMP+Duphalac® the sample with the highest degree of hydrolysis of lactose. In general, no significant differences ($p > 0.05$) were found for SMP samples with OsLu and GOS. Lactulose was significantly less susceptible to hydrolysis in SMP+Duphalac® than in SMP+OsLu. Furthermore, lactulose present in OsLu and Duphalac® was more prone to degradation than OS, probably ascribed to its lower Mw, although the difference was only significant after 1 h of digestion. Finally, OS were significantly more hydrolysed in SMP+GOS than in SMP+OsLu reaching values of 35% and 15%, respectively after 2 h; this was probably due to the more stable $\beta(1\rightarrow6)$ linkages in the OsLu mixture as compared to $\beta(1\rightarrow4)$ in GOS and the presence of fructose at the terminal end of molecule (Hernandez-Hernandez et al. 2012). These results indicate that OS (DP≥3) present in OsLu were scarcely affected by the gastrointestinal digestion under the conditions used in the present work, being digested in a very low proportion in the small intestine which would favour the presence of a OS in the distal portions of colon to be fermented by beneficial bacteria.

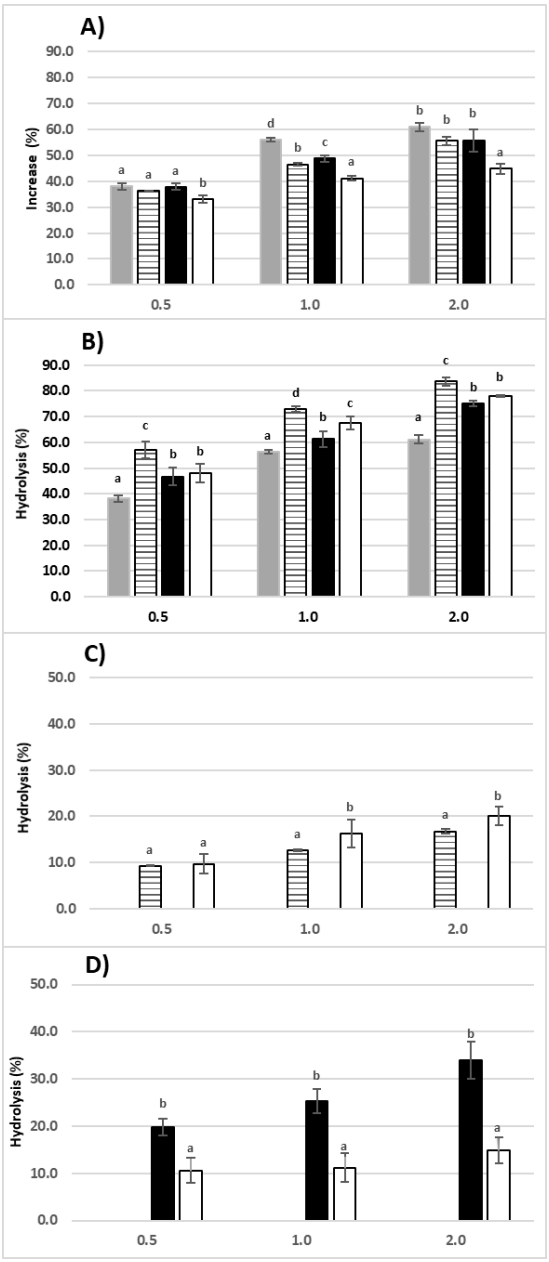

Figure 4. Evolution of carbohydrates over time during the gastric and intestinal digestion with RSIE. Figure shows the results for each fraction analyzed A) Monosaccharides, B) Lactose, C) Lactulose and D) Oligosaccharides after 0.5, 1.0 and 2.0 h of digestion. Grey bar represents SMP samples; Striped bar, SMP+Duphalac; Black bar, SMP+GOS and White bar, SMP+OsLu. The results are shown as percentage of increase (A) or hydrolysis (B, C, D) relatively to their respective controls. Results are presented as mean ± SD (n=4). Bar with different lower-case letters (a–d) represent statistical significant differences between each carbohydrate fraction at the same digestion time for their mean values at the 95.0 % confidence.

To the best of our knowledge this is the first *in vitro* study on the digestion of prebiotics derived from lactose and lactulose as ingredients in a real food. The results obtained underline those of Hernandez-Hernandez et al. (2012) who pointed out, in *in vivo* assays with rats, that mixtures of OsLu were less digested than GOS. Particularly, the trisaccharide fraction of the former was 13% digested in the ileum, whereas in the latter case digestion was close to 53%. In both cases, the studied samples were the corresponding enzymatic mixtures obtained by transglycosylation and the presence of other food components was not considered. The small differences found in the total hydrolysis values with respect of our results could be ascribed to the differences in the experimental conditions.

Conclusions:

According to the results obtained is possible to conclude that the presence of prebiotic carbohydrates in milk, at prebiotic doses, did not affect the gastric digestion of milk proteins, following the Infogest protocol. Similarly, under the same gastrointestinal digestion method, hardly any change was detected in the carbohydrate fraction of milk with GOS, Duphalac® and OsLu after 2 h of digestion. This might indicate the resistance of the three prebiotic mixtures, including OsLu, to gastric and pancreatic fluids and bile salts.

However, when the digested samples of milk with prebiotics were subjected to intestinal digestion by a small gut intestinal extract of rat a dissimilar behaviour in the three cases was observed, OsLu samples being the most resistant to the action of enzymes present in the rat intestine extract, mainly in the case of OS fraction. These results highlight the possibility of OsLu to reach the large intestine, target organ, to exert their potential prebiotic effects.

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Chapter 3

In vitro digestibility of galactooligosaccharides: Effect of the structural features on their intestinal degradation

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Abstract:

Small intestinal brush border membrane vesicles (BBMV) from pig were used to digest galactooligosaccharides from lactose (GOS) and from lactulose (OsLu). Dissimilar hydrolysis rates were detected after digestion. Predominant glycosidic linkages and monomeric composition affected the resistance to intestinal digestive enzymes. $\beta(1\rightarrow3)$ GOS mixture was the most susceptible to hydrolysis (50.2%), followed by $\beta(1\rightarrow4)$ (34.9%), whereas $\beta(1\rightarrow6)$ linkages were highly resistant to digestion (27.1%). Monomeric composition provided a better resistance in $\beta(1\rightarrow6)$ OsLu (22.8%) as compared to $\beta(1\rightarrow6)$-GOS (27.1%). This was also observed for β-galactosyl-fructoses and β-galactosyl-glucoses where the presence of fructose provided higher resistance to digestion. Thus, the resistance to small intestinal digestive enzymes highly depends on structure and composition of prebiotics. Increasing knowledge on this regard could contribute to the future synthesis of new mixtures of carbohydrates, highly resistant to digestion and with potential to be tailored prebiotic with specific properties, targeting, for instance, specific probiotic species.

Introduction:

Knowledge about the diversity of human microbiota and its relation to health has been largely gathered during last years. Moreover, there is a clear evidence suggesting that our microbiota is deeply implicated in a wide range of metabolic functions extending beyond the gut (Heinz-Buschart et al. 2018), such as, the regulation of the central nervous system homeostasis through immune, vagal and metabolic pathways (Carabotti et al. 2015; Forsythe et al. 2014; Sherwin et al. 2016) or the prevention of bone and respiratory diseases (Ibañez et al. 2019; Sozanska et al. 2019). One of the most used strategies to modulate the composition and metabolic activity of microbiota is the use of prebiotics (Moreno et al. 2017).

Prebiotics definition refers to a "substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al. 2017). These compounds are characterized by the resistance to the digestion and acid conditions in the upper gastrointestinal tract and the ability to reach the colon without alteration in their structure (Roberfroid et al. 2010). To date, although a considerable number of compounds have been proposed as potential prebiotics, all well-recognized prebiotics are carbohydrates, mainly inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS) and lactulose. Among these, GOS have attracted growing interest due to the

presence of galactose-based oligosaccharides, similar to those in human milk oligosaccharides (HMOs) (Sangwan et al. 2011).

GOS are commonly obtained by enzymatic synthesis from lactose by *β*-galactosidases and they are constituted by a complex mixture of galactoses linked by different linkages *β*(1→1), *β*(1→2), *β*(1→3), *β*(1→4) and *β*(1→6) and can vary from 1 to 8 units and a terminal glucose (Moreno et al. 2014). Composition of the obtained GOS mixture is deeply affected by several factors such as, the enzyme source, lactose concentration, substrate composition and reaction conditions (temperature, time and pH) (Moreno et al. 2014; Gänzle et al. 2012; Torres et al. 2010). Galactooligosaccharides derived from lactulose (OsLu) have been also proposed as emerging prebiotic compounds since they might provide enhanced prebiotic properties compared to conventional GOS by increasing short-chain fatty acids content and the population of *Bifidobacterium* and *Lactobacillus* species (Moreno et al. 2014; Hernández-Hernández et al. 2012). OsLu are obtained similarly to GOS using lactulose as substrate and are constituted by galactose units, linked by a variety of glycosidic linkages (*β*(1→6), *β*(1→1) and/or *β*(1→4)) determined by the enzyme source, and a terminal fructose (Díez-Municio et al. 2014).

The susceptibility of oligosaccharides to small intestinal digestion highly depends on their structure, compromising their absorption and digestion fate

(Gosling et al. 2010). However, ever since prebiotics were first defined, most of the investigations have been carried out focusing on their effect on the gut microbiota composition and/or activity, and few efforts have been made towards the study of the resistance of these compounds to digestion in the small intestine (Tanabe et al. 2014; Tanabe et al. 2015; Oku et al. 1984; Ohtsuka et al. 1990; Ferreira-Lazarte et al. 2017a; Ferreira-Lazarte et al. 2017b). Moreover, the standardized official methods to determine the digestibility of carbohydrates present several limitations, such as those related to the matrix composition of the sample should be limited in complexity, the lack of simulation of realistic enzyme substrate ratios and removal of digested products; but most importantly, they do not take into consideration the disaccharidases that are present in the small intestinal brush border membrane vesicles in mammals (Drechsler et al. 2018; Egger et al. 2016; McCleary et al. 2010). Recently, the use of mammalian intestinal enzymes has been reported as an excellent alternative method to determine carbohydrate digestion (Tanabe et al. 2015; Ferreira-Lazarte et al. 2017b; Strube et al. 2015).

In vivo and *in vitro* studies have described considerable digestion rates in the small intestine of different types of GOS in rats (15-53% hydrolysis degree after 2 h of digestion), (Hernández-Hernández et al. 2012; Ferreira-Lazarte et

al. 2017a; Ferreira-Lazarte et al. 2017b; Jantschen-Krenn et al. 2013; Marín-Manzano et al. 2013), questioning the general acceptance that these compounds reach intact the colon. These authors also have reported a different resistance to the upper gastrointestinal tract conditions as well as a different effect on microbiota depending on the main *β*-linkage in the mixture. Thus, *β*(1→6) linkages have been reported to be less prone to degradation by intestinal enzymes and to exert better prebiotic effect as compared to other *β*-linkages.

Bearing that in mind, the aim of the present study was to evaluate the digestibility of recognized prebiotics such as GOS, with predominant *β*(1→3), *β*(1→4) or *β*(1→6) linkages, as well as emerging prebiotic candidates derived from lactulose (OsLu, *β*(1→6)) using small intestinal brush border membrane vesicles from pig.

Materials and methods:

Chemicals and reagents. D-Galactose (Gal), D-glucose (Glc), sucrose (*β*-D-Fru(2→1)-α-D-Glc), trehalose (α-D-Glc(1→1)-α-D-Glc), lactulose (*β*-D-Gal(1→4)-D-Fru), phenyl-*β*-glucoside, *o*-nitrophenyl (*o*-NP), p-nitrophenyl (*p*-NP), *o*-nitrophenyl-*β*-D-galactopyranoside (*o*-NPG) and *p*-nitrophenyl-α-glucopyranoside (*p*-NPG) standards were obtained from Sigma-Aldrich (St

Louis, MO). Lactose (*β*-D-Gal(1→4)-D-Glc) was obtained from ACROS organics (Geel, Belgium) and fructose was obtained from Fluka analytical (St. Gallen, Switzerland). All standard carbohydrates were of analytical grade (purity ≥ 95%). *Kluyveromyces marxianus* cells were kindly provided by Professor Robert Rastall from The University of Reading (United Kingdom). Nutritive medium (peptone, lactose and yeast extract) were supplied by Sigma-Aldrich.

Small intestinal brush border membrane vesicles (BBMV) preparation. Small intestinal brush border vesicles from six post-weaned pigs (7-10 months old) were obtained following methodology previously reported (Kessler et al. 1978; Tanabe et al. 2015). Briefly, three pig small intestines, from the duodenum to the ileum, were obtained from a local slaughterhouse (Coca, Segovia, Spain). Immediately after sacrifice, the samples were kept at 4 ºC and transferred to the laboratory in less than 2 h. The small intestines were rinsed with cold phosphate buffered saline solution (PBS) (pH 7.3 – Oxoid; Basingstoke, UK), then slit open and scrapped with a glass slide. The mucose scrapped was suspended (1:1, w/v) in 50 mM mannitol dissolved in PBS at 4 ºC, homogenized during 10 min using a Ultra-Turrax® (IKA T18 Basic), adjusted with $CaCl_2$ to a final concentration of 10 mM and centrifuged at 3,000 *g* during 30 min. The supernatant was centrifuged at

27,000 *g* during 40 min and the resulting pellet, containing the BBMV, was re-suspended in buffer maleate (50 mM) pH 6.0 containing $CaCl_2$ (2 mM) and sodium azide (0.02%). Samples were lyophilized and kept at -80ºC.

Prebiotic oligosaccharides. OsLu were obtained at pilot plant scale by Innaves S.A. (Vigo, Spain) following the method described by López-Sanz et al. (2015). Briefly, OsLu were synthesized using a commercial lactulose preparation (670 g/L; Duphalac, Abbott Biologicals B.V., Olst, The Netherlands), and a commercial preparation including *β*-galactosidase from *Aspergillus oryzae* (16 U/mL; Sigma) at pH 6.5, 50 ºC and 350 rpm during 24 h. In addition, three different commercially available GOS mixtures with predominant *β*(1→3) linkages GOS (named GOS-1), predominant *β*(1→4) linkages GOS (named GOS-2) and predominant *β*(1→6) GOS (named GOS-3), were tested.

Prebiotic oligosaccharides purification. Purification of prebiotic compounds was carried out by yeast treatment with *K. marxianus*.

K. marxianus cells were grown in YPD (1 % (w/v) yeast extract, 2 % peptone and 2 % lactose) (500 mL) at 37 ºC during 48 h. Samples were then centrifuged at 4,000 *g* for 10 min and washed three times on PBS (500 mL), supernatant was discarded, and washed samples were taken to incubation. Twenty-five mL of prebiotic ingredients (10% in PBS) and *K. marxianus*

yeast (equivalent to 25 mL YPD) were incubated at 37 ºC for 48 h. Samples were then centrifuged at 4,000 x*g* for 20 min, filtered by 0.2 µm and then lyophilized and kept at -20ºC until analysis. Purification process was carried out three times for each sample (n=3) and monitored by GC-FID as explained below. GOS-1 mixture which was previously constituted by 30% monosaccharides, 22% lactose, 25% disaccharides and 23% trisaccharides (w:w) showed a loss of 67% monosaccharides after K. marxianus treatment. GOS-2 was composed by 22% monosaccharides, 19% lactose, 8% disaccharides, 44% trisaccharides and 7% tetrasaccharides (w:w) showing a 97.4 % decrease in monosaccharides composition. GOS-3 composition which was 38% monosaccharides, 14% lactose and 52% oligosaccharides (w:w) showed a decrease of 95% of monosaccharides. OsLu was constituted by 7.8% monosaccharides, 49.3% lactulose 28.8% disaccharides and 14.1% trisaccharides (w:w).

Small Intestinal BBMV characterization. Pig small intestinal BBMV (10 mg/mL) was homogenized in ice-cold 0.05 M sodium phosphate buffer solution and then centrifuged at 6,000 x*g* for 15 min. Supernatant was used as enzyme solution for determining protein content and enzymatic activity.

Protein content determination. Total protein content of the pig small intestinal BBMV was quantified according to the Bradford method, using the

Bio-Rad Protein Assay kit and bovine serum albumin as a standard. The absorbance was monitored at 595 nm (Bradford 1976).

Hydrolytic activities. ***β-galactosidase and maltase activities.*** The determination of the pig intestinal *β*-galactosidase activity was adapted from Warmerdam et al. (2014). Briefly, a solution of *o*-NPG (0.5 mg/mL) in phosphate buffer 0.05 M, pH 7.0 was prepared. The enzymatic activity was determined by incubating 1,900 µL of the *o*-NPG solution and 100 µL of enzyme solution from BBMV for 2 h at 37 °C. The method is based on the measurement of the continuous release of *o*-NP from *o*-NPG. The absorbance of released *o*-NP was measured at 420 nm every 30 s using a spectrophotometer (Specord Plus, Analytik Jena) together with a temperature controller (Jumo dTRON 308, Jumo Instrument Co.). The specific enzymatic activity (U) was expressed in µmol min^{-1} g^{-1}, where one unit was defined as the amount of enzyme that produced 1 µmol of *o*-NP in one min of reaction (biological replicates - n = 3). Similar procedure was used to determine the maltase activity by using a solution of *p*-NPG in phosphate buffer 0.05 M, pH 6.8 (0.05% w/w) and monitoring the release of *p*-NP at 420 nm every 20 s (n=3).

Sucrase and trehalase activities. Sucrase and trehalase activities were determined following a method described in a previous work (Ferreira-

Lazarte et al. 2017b). A solution of sucrose or trehalose (0.5% w/v) in sodium phosphate buffer 0.05 M, pH 6.5 was used. An eppendorf tube with 500 µL of sucrose or trehalose solution was preheated at the reaction temperature, 37 °C. Subsequently, 200 µL of enzyme solution was added and the mixture was incubated for 2 h and different aliquots were taken at different times (5, 10, 15, 30, 60, 90 and 120 min). Hydrolysis was stopped by adding 700 µL of a 3,5-dinitrosalicylic acid (DNS) solution. Sucrase and trehalase activity were determined measuring the reducing sugars released from the corresponding disaccharide hydrolysis at 540 nm, according to the DNS method (Miller et al. 1959). The specific enzymatic activity (U) was expressed in µmol min^{-1} g^{-1}, where one unit was defined as the amount of enzyme that produced 1 µmol of reducing sugars in one min of reaction (n=3).

***In vitro* digestion of prebiotic compounds with BBMV.** The digestibility of three different types of GOS, OsLu and lactose and lactulose was evaluated using BBMV. First, a solution of BBMV (10 mg/mL) in PBS solution, 6.8 pH, was prepared. Fifteen milliliters of this solution containing BBMVs (10 mg/mL) were placed in centrifuge tubes (two per sample) and prebiotic or disaccharides samples were added at a concentration of 0.2 mg/mL. Digestions were then initiated at 37 ºC during 5 h using 750 rpm in an orbital

Thermomixer comfort (Eppendorf®). Aliquots of 1 mL (x2) were taken at 0, 1, 2, 3, 4 and 5 h of digestion and immediately heated in boiling water for 5 min to stop the reaction.

Furthermore, incubation of BBMV without any carbohydrate source was also analyzed. Results showed quantifiable amounts of glucose as the digestion proceeded. These values were conveniently substracted to avoid any overestimation of the monosaccharide fraction.

Carbohydrates quantification by GC-FID. Carbohydrates present in the samples and digested mixtures were analysed as trimethylsilylated oximes (TMSO) by gas chromatography coupled to ionization flame detector (GC-FID) following the method of Brobst & Lott Jr, (1966). First, 500 μL of samples (0.1 mg carbohydrates) was added to 500 μL of phenyl-*β*-glucoside (Internal Standard, IS) and the mixture was dried in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). TMSO derivatives were formed by adding 250 μL of hydroxylamine chloride in pyridine (2.5% w/v) and heating the mixture at 70 ºC for 30 min, followed by the addition of hexamethyldisilazane (250 μL) and trifluoroacetic acid (25 μL) and incubated at 50 ºC for 30 min. Mixtures were centrifuged at 6,700 *g* for 2 min and supernatants were injected in the GC-FID. TMSO derivatives were separated using a fused silica capillary column DB-5HT (5%-phenyl-

methylpolysiloxane; 30m x 0.25mm x 0.10μm, Agilent). Nitrogen at 1 mL/min was used as carrier gas. Injector and detector temperatures were set at 280 and 385 ºC, respectively. The oven temperature was set from 150 ºC to 380 ºC at a ratio of 3 ºC/min. Data acquisition and integration were done using Agilent ChemStation software (Wilmington, DE, USA). Response factors were calculated after duplicate analysis of standard solutions (fructose, glucose, galactose, lactose, lactulose and raffinose) over the expected concentration range in samples, (0.005–1 mg) and IS (0.25 mg).

Statistical Analysis. Statistical analysis was carried out using SPSS for Windows, version 23.0. One-way analysis of variance (ANOVA) and Tukey's *post hoc* test was used to determine significant differences ($p < 0.05$) between concentrations of carbohydrates in each prebiotic sample (n=3).

Results and discussion:

High decreases in monosaccharide composition was observed after *K. Marxianus* (Material and methods section 2.3.1). In this sense, monosaccharides are the major impurities in GOS obtainment, therefore, removal of these compounds is recommended mainly due to their undesirable caloric value and glycaemic index (Gosling et al. 2010). Furthermore,

inhibition of β-galactosidase by glucose and galactose in transgalactosylation and hydrolysis reaction of carbohydrates was reported (Vera et al. 2011).

BBMV enzymatic characterization. The brush border of the mammalian intestinal mucosa contains several key enzymes present as multienzyme complexes, i.e. sucrase-isomaltase, lactase-phlorizin hydrolase, maltase-glucoamylase and trehalase (Feher 2012). Accordingly, it is well reported the presence of those carbohydrases in the brush border of the intestinal mucosa of pig (Tanabe et al. 2015; Kidder et al. 1978; Rubio et al. 2014). **Table 1** shows the protein content and main enzymatic activities (β-galactosidase, maltase, sucrase and trehalase) of BBMV measured under the assayed digestion conditions. Maltase activity (753.1 U/g) was the highest with ten-fold higher values than the other measured activities. This higher value can be ascribed to the multiple maltase activities carried out by different enzymatic complexes such as maltase-glucoamylase which has two catalytic sites able to hydrolyse maltose. This maltase activity is also present in both catalytic sites found in the complex sucrase-isomaltase. Both enzymatic complexes are the most abundant glycosidases in the small intestine (Hooton et al. 2015). To date, some studies have characterized the carbohydrase activities of small intestinal enzymes in pigs (Tanabe et al. 2015; Rubio et al. 2014; Gnoth et al. 2000; Sørensen et al 1982), showing a clear predominance

of maltase activity as compared to other activities, which agrees with the data obtained in this work.

Table 1. Specific enzymatic activities and protein content of Small Intestinal BBMV.

Activity	Substrate	Conditions	U (µmol/min g)
β-galactosidase	*o*-NPG	7.0; 37 °C	70.1 ± 1.4
Maltase	*p*-NPG	6.8; 37 °C	753.1 ± 16.5
Sucrase	Sucrose	6.8; 37 °C	19.9 ± 2.2
Trehalase	Trahalose	6.8; 37 °C	21.4 ± 7.6

Protein content of BBMV was 7.3 ± 0.5 %
Data are expressed as means ± SD (n = 3)

Digestion of prebiotic carbohydrates by BBMV. Figure 1 shows GC-FID profiles of oligosaccharides before and after 5h of digestion with BBMV. Differences were observed between the profiles of the three GOS mixtures, 1,4-galactobiose (*β*-Gal-(1→4)-Gal) and 1,6-galactobiose (*β*-Gal-(1→6)-Gal) were identified as peaks 2 and 5, respectively in all samples. *β*-Gal-(1→3)-Glc and allolactose (*β*-Gal-(1→6)-Glc), both isomers of lactose were also detected in all samples as peaks 3 and 4, respectively. Further structural differences were found in the trisaccharides fraction. *β*-1,4-Galactosyl-lactose (*β*-Gal-(1→4)-*β*-Gal-(1→4)-Glc, peak 6) was detected in all samples, *β*-1,6-galactosyl-lactose (*β*-Gal-(1→6)-*β*-Gal-(1→4)-Glc, peak 8) was found in GOS-2 and GOS-3 samples and *β*-1,3-galactosyl-lactose (*β*-Gal-(1→3)-*β*-Gal-(1→4)-Glc, peak 7) was only detected in GOS-1 mixture.

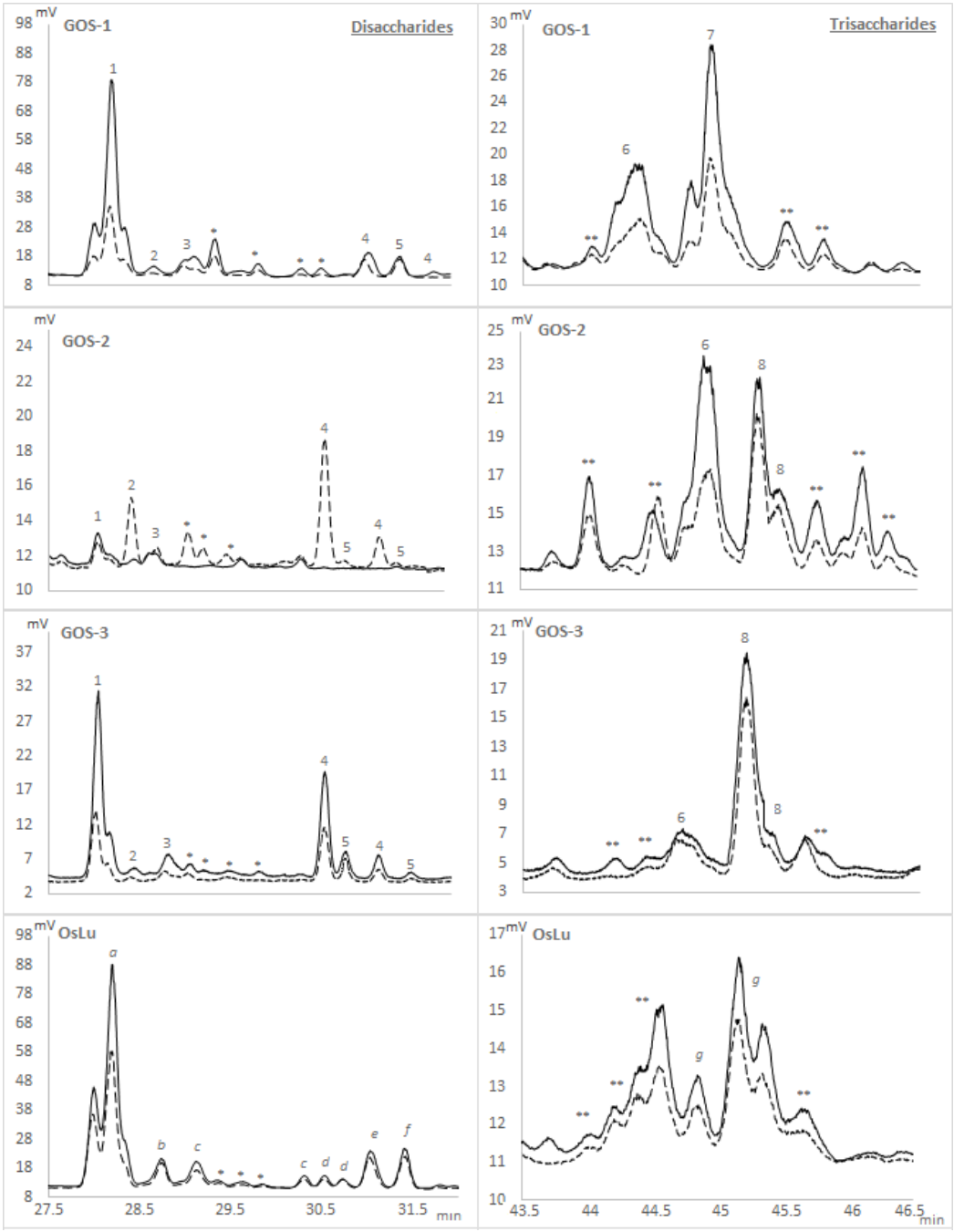

Figure 1. Chromatographic profiles of TMSO derivatives of prebiotics oligosaccharides before (continuous line) and after 5 h of digestion with BBMV (striped line). GOS Disaccharides: 1, lactose; 2, 1,4-galactobiose; 3, β-Gal-(1→3)-Glc; 4, allolactose; 5, 1,6-galactobiose and * other disaccharides. GOS Trisaccharides: 6, β-Gal-(1→4)-β-Gal-(1→4)-Glc; 7, β-Gal-(1→3)-β-Gal-(1→4)-Glc; 8, β-Gal-(1→6)-β-Gal-(1→4)-Glc and ** other trisaccharides. OsLu Disaccharides: a, lactulose; b, 1,4-galactobiose; c, 1,2-galactobiose+1,3-galactobiose; d, β-Glc-(1→6)-Fru; e, β-Glc-(1→1)-Fru; f, , 1,6-galactobiose and * other disaccharides. OsLu trisaccharides: g, β-Gal-(1→6)-β-Gal-(1→4)-Fru and ** other trisaccharides.

Tetrasaccharides were also noticed in GOS-2 mixture (**Table 2)** and this fraction was mainly constituted by *β*-Gal-(1→4)-*β*-Gal-(1→4)-*β*-Gal-(1→4)-Glc and other tetrasaccharides not identified in this work (Hernández-Hernández et al. 2012; Ruiz-Matute et al. 2012; Moreno et al. 2014).

OsLu mixture was constituted by *β*(1→6) as the main glycosidic linkage and mostly by galactosyl galactoses (Gal-Gal) and galactosyl fructoses (Gal-Fru). *β*-(1→6)-galactosyl-lactulose (*β*-Gal-(1→6)-*β*-Gal-(1→4)-Fru) was identified as the main trisaccharide in the sample. In general, all assessed GOS and OsLu showed a diminution after the BBMV digestion, although considerable differences among all studied samples were observed.

Tables 2 and 3 show the quantitative determination of individual carbohydrates in GOS and OsLu during digestion, respectively. A progressive increase in the level of monosaccharides was found in all samples as digestion proceeded, which was concomitant with the decrease in di- and trisaccharide fractions. Digestion of standard solutions of lactose or lactulose with BBMV is also shown for comparative purposes. As expected, lactose was much more prone to degradation than lactulose due to the presence of fructose instead of glucose in the *β*-linkage of the latter (Olano & Corzo 2009).

Table 2. Carbohydrate content (mg/100 mg of total carbohydrates) determined by GC-FID analysis in GOS samples during the digestion with pig small intestinal brush border membrane vesicles at 37 °C, pH 6.8

Digestion time (h)	Galactose	Glucose	Lactose	β(1→4) Gb	β(1→3) Gal-Glc	β(1→6) Gb	Allolactose	Other Disaccharides	β(1→4) Gal-la	β(1→3) Gal-la	β(1→6) Gal-la	Other Trisaccharides	Σ DI	Σ TRI	Σ TETRA	OS[a]
Lactose																
0	0.7 ± 0.0	1.1 ± 0.0	98.2 ± 0.0	-	-	-	-	-	-	-	-	-	-	-	-	-
1	26.7 ± 4.5	17.3 ± 7.0	56.1 ± 7.2	-	-	-	-	-	-	-	-	-	-	-	-	-
2	37.1 ± 6.3	28.9 ± 0.7	34.0 ± 2.7	-	-	-	-	-	-	-	-	-	-	-	-	-
3	31.5 ± 7.8	38.4 ± 11.5	30.0 ± 11.0	-	-	-	-	-	-	-	-	-	-	-	-	-
4	39.8 ± 7.7	41.9 ± 2.4	18.3 ± 6.6	-	-	-	-	-	-	-	-	-	-	-	-	-
5	47.4 ± 4.5	49.8 ± 5.1	2.8 ± 0.4	-	-	-	-	-	-	-	-	-	-	-	-	-
GOS-1																
0	5.5 ± 0.0	5.5 ± 0.0	34.2 ± 0.0	1.3 ± 0.0	5.4 ± 0.0	2.5 ± 0.0	4.8 ± 0.0	8.3 ± 0.0	9.6 ± 0.0	15.5 ± 0.0*	-	7.4 ± 0.0	22.4 ± 0.0	32.4 ± 0.0	-	54.8 ± 0.0
1	13.1 ± 3.5	15.7 ± 3.7	24.9 ± 2.4	1.3 ± 0.5	4.5 ± 1.1	2.7 ± 0.8	4.8 ± 0.7	6.0 ± 3.1	7.8 ± 1.1	12.0 ± 3.1*	-	7.1 ± 0.6	19.3 ± 4.1	26.9 ± 2.0	-	46.2 ± 6.1
2	18.2 ± 3.0	16.6 ± 3.0	23.9 ± 1.9	1.3 ± 0.3	4.2 ± 0.3	2.1 ± 0.6	4.7 ± 0.6	6.1 ± 0.7	8.1 ± 3.0	8.7 ± 1.1*	-	6.2 ± 0.7	18.3 ± 0.8	23.0 ± 2.6	-	41.3 ± 1.8
3	23.6 ± 3.2	29.9 ± 7.1	20.8 ± 2.2	1.1 ± 0.4	3.5 ± 0.3	2.3 ± 0.5	4.1 ± 2.3	6.1 ± 2.4	4.4 ± 1.0	9.1 ± 4.6*	-	4.9 ± 1.6	17.3 ± 4.5	18.4 ± 7.2	-	35.7 ± 11.5
4	29.0 ± 1.2	21.5 ± 2.2	17.2 ± 1.2	1.2 ± 0.1	3.2 ± 0.3	2.1 ± 0.6	3.6 ± 0.6	4.9 ± 0.1	3.3 ± 0.1	8.2 ± 0.3*	-	5.9 ± 0.7	15.0 ± 0.6	17.4 ± 1.0	-	32.3 ± 1.2
5	33.1 ± 4.0	28.9 ± 0.6	10.7 ± 2.1	1.2 ± 0.2	2.8 ± 0.3	2.1 ± 0.3	3.3 ± 0.2	3.2 ± 0.5	3.1 ± 0.1	6.0 ± 0.5*	-	4.7 ± 0.1	12.6 ± 0.2	14.7 ± 0.4	-	27.3 ± 0.7
GOS-2																
0	0.6 ± 0.0	0.0 ± 0.0	1.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.0	20.4 ± 0.0	-	18.2 ± 0.0	38.5 ± 0.0	4.1 ± 0.0	77.1 ± 0.0	16.4 ± 0.0	97.6 ± 0.0
1	8.6 ± 1.2	6.9 ± 0.2	1.7 ± 0.1	2.4 ± 0.6	0.9 ± 0.5	0.4 ± 0.1	3.2 ± 0.5	2.9 ± 0.6	15.0 ± 0.9	-	16.2 ± 1.0	26.9 ± 1.1	9.9 ± 0.7	58.1 ± 3.2	14.9 ± 0.5	82.9 ± 1.2
2	13.5 ± 1.0	9.5 ± 1.1	1.6 ± 0.5	2.2 ± 0.2	1.1 ± 0.1	0.4 ± 0.2	5.0 ± 0.9	3.1 ± 1.1	13.3 ± 1.3	-	15.6 ± 0.9	25.3 ± 0.9	11.7 ± 1.1	54.1 ± 2.0	12.8 ± 1.1	78.6 ± 0.9
3	16.0 ± 0.7	10.9 ± 0.2	1.3 ± 0.4	2.1 ± 0.5	1.2 ± 0.2	0.3 ± 0.3	5.8 ± 1.0	3.0 ± 0.5	10.5 ± 0.7	-	14.3 ± 0.7	23.7 ± 1.3	12.5 ± 0.4	48.5 ± 1.5	10.8 ± 1.3	71.8 ± 1.3
4	18.3 ± 0.3	12.3 ± 0.5	1.2 ± 0.3	2.4 ± 1.1	1.2 ± 0.3	0.3 ± 0.1	6.8 ± 0.7	3.9 ± 0.4	9.7 ± 0.4	-	14.1 ± 0.5	18.4 ± 0.8	14.5 ± 1.2	42.1 ± 0.9	10.5 ± 0.9	67.2 ± 0.7
5	21.2 ± 1.5	13.4 ± 1.0	0.9 ± 0.1	2.5 ± 0.8	1.0 ± 0.4	0.4 ± 0.2	8.0 ± 0.8	3.3 ± 0.2	8.8 ± 1.1	-	13.5 ± 0.3	16.1 ± 0.7	15.1 ± 0.9	38.4 ± 1.4	10.1 ± 1.1	63.5 ± 1.6
GOS-3																
0	0.7 ± 0.0	1.1 ± 0.0	25.1 ± 0.0	1.6 ± 0.0	4.1 ± 0.0	2.6 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.4 ± 0.0	-	32.1 ± 0.0	5.3 ± 0.0	26.4 ± 0.0	46.8 ± 0.0	-	73.1 ± 0.0
1	5.9 ± 1.6	4.5 ± 1.0	20.1 ± 1.5	1.1 ± 0.1	3.1 ± 0.1	2.9 ± 0.2	9.2 ± 1.0	6.6 ± 0.2	8.2 ± 1.3	-	31.0 ± 2.2	6.2 ± 1.0	22.9 ± 1.4	45.4 ± 2.6	-	68.3 ± 1.2
2	10.3 ± 0.9	8.5 ± 1.7	16.1 ± 1.5	1.0 ± 0.0	2.5 ± 0.1	3.1 ± 0.3	9.2 ± 0.3	6.4 ± 0.2	7.7 ± 1.5	-	28.9 ± 1.7	6.2 ± 0.7	22.2 ± 0.2	42.8 ± 2.3	-	65.1 ± 2.2
3	13.7 ± 0.4	11.0 ± 0.1	11.6 ± 0.6	0.9 ± 0.1	2.4 ± 0.4	3.0 ± 0.2	8.7 ± 1.1	6.2 ± 0.6	7.1 ± 1.5	-	29.2 ± 1.1	6.2 ± 0.7	21.2 ± 0.8	42.5 ± 0.6	-	63.7 ± 0.4
4	18.3 ± 1.2	15.6 ± 0.5	9.8 ± 0.4	0.9 ± 0.2	2.2 ± 0.3	2.9 ± 0.1	8.3 ± 2.0	5.5 ± 0.2	6.7 ± 0.2	-	25.1 ± 1.0	4.7 ± 0.6	19.8 ± 1.7	36.5 ± 1.3	-	56.3 ± 0.9
5	21.9 ± 0.3	17.0 ± 1.2	7.9 ± 0.1	0.9 ± 0.1	2.0 ± 0.5	3.4 ± 0.0	6.8 ± 0.2	4.6 ± 0.7	5.1 ± 0.5	-	26.7 ± 1.1	3.7 ± 0.0	17.8 ± 0.4	35.5 ± 1.2	-	53.3 ± 1.6

Data are expressed as the mean ± SD (n = 4).
Gb = galactobiose (β-Gal-(1→4/6)-Gal)
Gal-la = galactosyl-lactose (β-Gal-(1→4/6)-β-Gal-(1→4)-Glc)
[a] Oligosaccharides content based on the sum of di-, tri- and tetrasaccharides.
*Represents the peak constituted mainly by β-1,3 galactosyl-lactose and traces of β-1,6 galactosyl-lactose

Table 3. Carbohydrate content (mg/100 mg of total carbohydrates) determined by GC-FID analysis in OsLu samples during the digestion with pig small intestinal brush border membrane vesicles at 37 °C, pH 6.8

Digestion time (h)	Fructose	Galactose	Lactulose	β(1→4) Gb	β(1→3) β(1→2) Gb	β(1→6) Glc-Fru	β(1→1) Gal-Fru	β(1→6) Gb	Other Disaccharides	β(1→6) Gal-lu	Other Trisaccharides	Σ DI	Σ TRI	OS[a]
Lactulose														
0	5.1 ± 0.0	5.5 ± 0.0	89.5 ± 0.0	-	-	-	-	-	-	-	-	-	-	-
1	8.1 ± 3.2	8.0 ± 2.5	83.9 ± 4.2	-	-	-	-	-	-	-	-	-	-	-
2	10.4 ± 2.7	11.2 ± 2.2	78.4 ± 4.1	-	-	-	-	-	-	-	-	-	-	-
3	11.9 ± 1.0	14.2 ± 1.0	74.0 ± 2.4	-	-	-	-	-	-	-	-	-	-	-
4	15.2 ± 2.0	16.7 ± 1.3	68.1 ± 3.1	-	-	-	-	-	-	-	-	-	-	-
5	18.1 ± 0.4	18.9 ± 0.3	63.0 ± 0.5	-	-	-	-	-	-	-	-	-	-	-
OsLu														
0	1.2 ± 0.0	6.6 ± 0.0	49.3 ± 0.0	6.9 ± 0.0	5.7 ± 0.0	2.9 ± 0.0	5.5 ± 0.0	5.3 ± 0.0	2.5 ± 0.0	8.2 ± 0.0	5.9 ± 0.0	28.8 ± 0.0	14.1 ± 0.0	42.9 ± 0.0
1	2.3 ± 0.2	12.5 ± 2.1	44.0 ± 0.6	6.4 ± 0.3	5.4 ± 0.9	3.1 ± 0.0	5.6 ± 0.2	5.4 ± 0.2	2.2 ± 0.3	7.9 ± 0.8	5.4 ± 0.1	28.1 ± 1.1	13.2 ± 0.5	41.2 ± 1.5
2	3.4 ± 0.2	17.5 ± 0.3	40.4 ± 1.5	5.9 ± 0.3	5.1 ± 1.0	2.9 ± 0.8	5.2 ± 0.2	4.9 ± 0.1	2.0 ± 0.7	7.4 ± 1.1	5.1 ± 0.3	25.9 ± 1.0	12.5 ± 0.1	38.4 ± 1.0
3	4.7 ± 0.4	20.5 ± 1.1	37.5 ± 1.0	5.6 ± 0.6	4.6 ± 0.3	2.8 ± 0.1	5.1 ± 0.1	4.9 ± 0.1	2.0 ± 0.1	7.3 ± 0.2	5.0 ± 0.1	25.0 ± 0.6	12.3 ± 0.3	37.3 ± 0.8
4	5.0 ± 0.5	24.5 ± 0.5	35.7 ± 0.5	5.4 ± 0.2	4.5 ± 0.7	2.5 ± 0.5	4.7 ± 0.1	4.5 ± 0.2	1.7 ± 0.5	7.1 ± 0.5	4.4 ± 0.6	23.3 ± 0.5	11.4 ± 0.9	34.7 ± 0.9
5	7.1 ± 0.2	26.7 ± 2.4	33.1 ± 1.5	5.6 ± 0.6	4.3 ± 0.3	2.6 ± 0.1	4.6 ± 0.1	4.5 ± 0.1	1.1 ± 0.0	6.6 ± 0.8	4.0 ± 0.9	22.6 ± 0.5	10.5 ± 1.3	33.1 ± 1.4

Data are expressed as the mean ± SD (n = 4).
Gb = galactobiose (β-Gal-(1→1/2/3/4/6)-Gal)
Gal-lu = galactosyl-lactulose (β-Gal-(1→6)-β-Gal-(1→4)-Fru)
[a] Oligosaccharides content based on the sum of di- and trisaccharides.

Lactose degradation in GOS samples was remarkably lower (50-68 %) when compared to the standard solution (97 %) (**Table 1S, *Annex B*)**, probably due to the fact that the degradation of particular GOS trisaccharides or tetrasaccharides could revert released lactose, as well as to the presence of other carbohydrates in the GOS mixtures which might mitigate the straightforward digestion of lactose when is present alone. Regarding lactulose digestion, the standard solution showed a slight lower hydrolysis than that observed for lactulose present in OsLu (29.5 and 32.8 %, respectively, after 5 h of digestion). Similar behaviour was obtained in a previous work comparing the digestibility of prebiotics added to milk in an *in vitro* study with a small intestine rat extract (Ferreira-Lazarte et al. 2017a). Concerning disaccharides degradation, β-Gal-(1→3)-Glc and β-Gal-(1→6)-Glc (allolactose) exhibited a slight decrease in their content after the BBMV digestion. Allolactose (β(1→6)) was the most resistant to hydrolysis when compared to lactose (β(1→4)) and β(1→3) structures. In this regard, it has been previously reported the high resistance of allolactose to intestinal mucosa with less than 5% of hydrolysis compared with lactose in an *in vitro* human assay (Burvall et al. 1980) and in an *in vivo* study with rats (Hernández-Hernández et al. 2012). Concerning galactosyl galactoses, none of these carbohydrates provided any noticeable change, indicating their stability during the digestion with BBMV. Indeed, an increase of these

compounds was found in some samples. Specifically, GOS-2 mixture showed an increase of 4' and 6'-galactosyl galactose, respectively, suggesting the possible breakdown of the $\beta(1\rightarrow4)$ linkage of the terminal glucose in their trisaccharide fraction. Regarding OsLu disaccharides, high resistance of galactosyl galactoses was also observed. Limited hydrolysis of galactosyl-fructoses was found, with $\beta(1\rightarrow6)$-galactosyl-fructose linkages as the lowest decrease among all determined disaccharides (**Table 3**). According to Hernandez-Hernandez et al. (2012), it is plausible that, in a similar way to lactulose, other galactosyl-fructoses can be highly resistant to digestion within the mammalian small intestinal system. In line with our results, Julio-Gonzalez et al. (2019) have recently reported the potential higher resistance to mammalian digestion of galactosyl-galactoses than galactosyl-glucoses.

Regarding trisaccharides fraction, data in **Table 2** shows that $\beta(1\rightarrow3)$-galactosyl-lactose in GOS-1 exhibited a higher hydrolysis than $\beta(1\rightarrow4)$-galactosyl-lactose in GOS-2 and $\beta(1\rightarrow6)$-galactosyl-lactose in GOS-3. However, to provide more insight into the effect on linkage on trisaccharides fraction, **Table 4** shows the hydrolysis degree of each different linkage trisaccharide present in all samples. In addition, the slope of the representation of hydrolysis degree (%) *vs* time (h), which could be considered as the hydrolysis rate, is also shown.

Table 4. Hydrolysis degree (%) evolution of different linkage trisaccharides (Tri) in each sample during the *in vitro* digestion with BBMV.

	GOS-1		GOS-2		GOS-3		OsLu
Digestion time (min)	β(1→3) Gal-la	β(1→4) Gal-la	β(1→4) Gal-la	β(1→6) Gal-la	β(1→4) Gal-la	β(1→6) Gal-la	β(1→6) Gal-lu
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	22.6	18.8	26.5	11.0	12.8	3.4	3.7
2	43.9	15.6	34.8	14.3	18.1	10.0	9.8
3	41.3	54.2	48.5	21.4	24.5	9.0	11.0
4	47.1	65.6	52.5	22.5	28.7	21.8	13.4
5	61.3	67.7	56.9	25.8	45.7	16.8	19.5
Hydrolysis degree slope (after 2h)	**21.9**	**7.8**	**17.4**	**7.1**	**9.0**	**5.0**	**4.9**

Taking into account a standard intestinal digestion time of 2 h, the hydrolysis degree of trisaccharides showed *β*(1→3)-galactosyl-lactose (hydrolysis rate of 21.9% as determined in GOS-1) to be more prone to degradation by intestinal enzymes followed by *β*(1→4)-galactosyl-lactose (7.8-17.4%), whereas *β*(1→6)-galactosyl-lactose (5.0-7.1%) and *β*(1→6)-galactosyl-lactulose (4.9%) exhibited the highest resistance to hydrolysis.

Concerning oligosaccharides as a whole (that is, the sum of di, tri and tetrasaccharides), the linkages *β*(1→6), abundant in GOS-3 and OsLu, demonstrated to be the most resistant to intestinal degradation (**Figure 2, Table 1S, *Annex B*)**, where the presence of fructose at the reducing end of

molecules provided OsLu a slight better resistance to digestion with 22.8 % against 27.1 % of hydrolysis for GOS-3 after 5 h (**Figure 2C**).

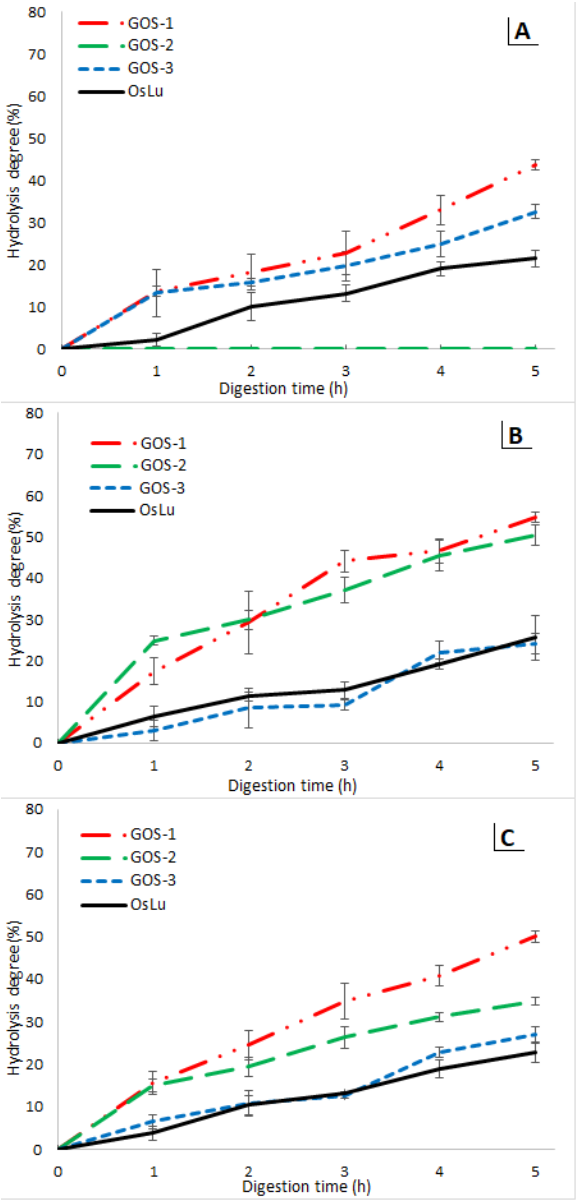

Figure 2. Evolution of hydrolysis (%) of carbohydrates during digestion with small intestine BBMV from pig at 37 ºC, pH 6.8. Disaccharides (A), trisaccharides (B) and oligosaccharides (C) expressed as the sum of di-, trisaccharides and tetrasaccharides in GOS-2

Furthermore, hydrolysis rate for GOS-3 and OsLu (**Table 5**) showed a lower degradation for OsLu as compared to GOS-3 after 2 and 5 h of digestion. GOS-2 oligosaccharides mixture was slightly more prone to degradation (34.9 %) with a higher hydrolysis rate after the BBMV digestion whereas GOS-1 oligosaccharides mixture exhibited the highest degree of hydrolysis with 50.1 % (**Table 1S)** degradation and the highest hydrolysis rate after 2 h (12.3) and 5 h (9.6) of treatment with BBMV from pig small intestine as compared to the other samples (**Table 5)**.

Table 5. Hydrolysis rate of oligosaccharides* in all samples during the *in vitro* digestion with BBMV.

	GOS-1 OS	GOS-2 OS*	GOS-3 OS	OsLu OS
Hydrolysis rate (after 5h)	9.6	6.6	5.3	4.6
Hydrolysis rate (after 2h)	12.3	9.7	5.5	5.2

* Expressed as the sum of di-, tri- and tetrasaccharides in GOS-2.

In this sense, a recent work highlighted the utility of a similar BBMV from pig small intestine to produce prebiotic GOS, and revealed that BBMV preferably synthesizes GOS linked by $\beta(1\rightarrow3)$ bonds, finding β-Gal-(1→3)-β-Gal-(1→4)-Glc as the main trisaccharide after comprehensive NMR analysis (Julio-Gonzalez et al. 2019). This study also pointed out no presence

of *β*-Gal-(1→6)-*β*-Gal-(1→4)-Glc, whereas the *β*-Gal-(1→4)-*β*-Gal-(1→4)-Glc trisaccharide was present but only at trace amounts. These findings support the data obtained in the current work since the most abundant glycosidic linkages, formed when mammalian intestinal β-galactosidase act as transgalactosidase, are expected to be preferentially broken under hydrolytic conditions.

In the other hand, regarding monosaccharides release, galactose amounts were slightly higher compared to glucose release, probably due to the composition of the main oligosaccharides in the samples. **Table 2** showed that the highest hydrolysis of GOS-1 oligosaccharides produced a higher release of total monosaccharides (62 mg/100 mg of total carbohydrates) after 5 h of digestion as compared to GOS-2, GOS-3 and OsLu (34.6, 38.9 and 33.8 mg/100 mg total carbohydrates, respectively). In this sense, the highest resistance of galactobioses and galactosyl-fructoses could affect positively to regulate the caloric intake and diminish the possible absorption of free monosaccharides in the small intestine, highlighting the key role of the monomer composition and type of glycosidic linkage in prebiotic oligosaccharide samples.

Results obtained in this work have demonstrated that the use of small intestinal BBMV from pig is a reliable and useful strategy to evaluate

prebiotic carbohydrate digestibility. Intestinal *in vitro* digestion with BBMV revealed the partial degradation of recognized prebiotics such as lactulose, different mixtures of GOS and an emerging prebiotic OsLu at considerably dissimilar levels. Our findings have revealed a stronger resistance of $\beta(1\rightarrow6)$ linkages oligosaccharides to *in vitro* digestion when compared to $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ linkages GOS. In general, $\beta(1\rightarrow3)$ followed by $\beta(1\rightarrow4)$ linkages were more prone to small intestinal degradation using BBMV. This less resistance to intestinal digestion was also found for galactosyl-glucose disaccharides as compared to galactosyl-galactoses (galactobioses). The key role of monomer composition was also underlined by the presence of fructose in OsLu mixture, providing, thus, a higher resistance to digestion of galactosyl-fructoses. Findings described in this work could be extrapolated to humans providing evidence on the structure-function relationship, as well as an increase on the knowledge of the different resistance of β-linkages for the sake of a future potential development of new tailored prebiotics. Moreover, the observed hydrolysis with mammalian small intestinal enzymes of recognized prebiotics could challenge the general belief that these compounds reach the colon without any alterations in their structure. More investigation should be done in order to gain more insight in the concept of prebiotics' digestibility.

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Part II: *In vitro* digestibility and fermentability of pectin and pectic compounds obtained from agricultural by-products

Chapter 4: *In vitro* fermentation properties of pectins and enzymatic-modified pectins obtained from different renewable bioresources

Chapter 5: Behaviour of citrus pectin during its gastrointestinal digestion and fermentation in a dynamic simulator (simgi®)

Chapter 4

In vitro fermentation properties of pectins and enzymatic-modified pectins obtained from different renewable bioresources

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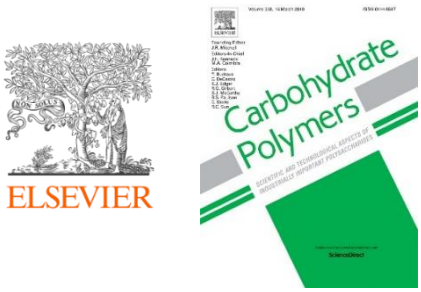

Abstract:

The suitability of artichoke and sunflower by-products as renewable sources of pectic compounds with prebiotic potential was evaluated by studying their ability to modulate the human faecal microbiota *in vitro*. Bacterial populations and short-chain fatty acid (SCFA) production were measured. Reduction of the molecular weight of artichoke pectin resulted in greater stimulation of the growth of *Bifidobacterium*, *Lactobacillus* and *Bacteroides/Prevotella*, whilst this effect was observed only in *Bacteroides/Prevotella* for sunflower samples. In contrast, the degree of methoxyl esterification did not have any impact on fermentability properties or SCFA production, regardless of the origin of pectic compounds. Although further *in vivo* studies should be conducted, either pectin or enzymatically-modified pectin from sunflower and artichoke by-products might be considered as prebiotic candidates for human consumption showing similar ability to promote the *in vitro* growth of beneficial gut bacteria as compared to well-recognized prebiotics such as inulin or fructo-oligosaccharides.

Introduction:

One of the most complex polysaccharides that exist in the cell wall of all higher plants is pectin (Kačuráková, et al. 2000). Pectin is not a single structure and comprises of a family of plant cell wall polysaccharides that contain galacturonic acid (GalA) linked at α-1,4 positions. It mainly consists of a GalA-rich backbone, known as homogalacturonan (HG ≈ 65%) which is partially methyl-esterified in C-6 and O-acethyl-esterified in positions 2 and 3 (Mohnen 2008). Rhamnose residues interrupt the HG structure to form rhamnogalacturonan I (RG-I ≈ 20-35%) which is based on a backbone consisting of a repeating disaccharide of GalA and rhamnose residues. In addition, some rhamnose residues may contain sidechains consisting of α-L-arabinose and/or β-D-galactose (arabinans, galactans and arabinogalactans). RG-II constitutes ≈ 2-10% of pectin and is the most complex, but is also believed to be the most conserved part of pectin molecules. RG-II has a HG backbone and is branched with rhamnose and other minor sugars such as fucose, glucuronic acid and methyl esterified glucuronic acid among other rare carbohydrates such as apiose, 2-*O*-methylxylose, and 2-*O*-methylfucose (Holck et al. 2014; Noreen et al. 2017).

The biological effects of pectins have been mainly studied on *in vitro* assays and they are highly fermentable dietary fibres. Furthermore, pectic-

oligosaccharides (POS) have been proposed as a new class of prebiotics capable of exerting a number of health-promoting effects (Olano-Martin et al. 2002). These benefits include a desirable fermentation profile in the gut (Gómez et al. 2016), potential *in vitro* anti-cancer properties (Maxwell et al. 2015), potential for cardiovascular protection (Samuelsson et al. 2016), as well as antibacterial, anti-inflammatory and antioxidant properties, among others (Míguez et al. 2016). Nevertheless, the details of the underlying mechanisms are still largely unknown and additional studies are needed on the structure-function interrelationship, as well as on the claimed effects caused by POS in humans (Gullón et al. 2013).

Apart from POS, whose degree of polymerization range from 3 to 10, during the past few years there has been a flourishing interest towards pectin derivatives, especially the so-called "modified pectins" (MP), a term standing for pectin-derived, water-soluble polysaccharide of lower molecular weight (Mw) than the original pectin and, normally, produced from citrus peel and pulp (Holck et al. 2014). These compounds can be obtained from pectins in their native form using chemical and enzymatic treatments, which produce lower Mw HG and fragments enriched in RG (Morris et al. 2013). The break-down of pectins not only leads to modification of their physico-chemical and

gelling properties (Ngouémazong et al. 2015), but also modulation of their bioactivity (Morris et al. 2013).

There are several *in vitro* and *in vivo* studies on the ability of MP to inhibit tumour growth and metastasis (Morris et al. 2013; Nangia-Makker et al. 2002; Park et al. 2017). Citrus MP inhibits *in vitro* and *in vivo* angiogenesis in different types of cancer by blocking the association of galectin-3 to its receptors (Zhang et al. 2015). Other beneficial health properties might include the reduction of atherosclerotic lesions (Lu et al. 2017), anti-inflammatory and antioxidant properties (Popov & Ovodov 2013; Ramachandran et al. 2017) or immunostimulatory properties (Vogt et al. 2016). However, most of these studies were performed using cell cultures or in mice and extrapolation of the results to human or clinical investigations should be considered with caution.

Nonetheless, only a few recent studies have addressed the prebiotic potential of MP in terms of the fermentation properties. A slight or no increase was observed in the faecal lactobacilli count during an *in vivo* study with rats fed with citrus MP (Odun-Ayo et al. 2017). Di et al. (2017) compared five structurally different citrus pectic samples (3 of them were POS and 2 were MP) and found that two POS and one MP exhibited bifidogenic effects with similar fermentabilities in human faecal cultures. These authors concluded

that Mw and degree of methoxylation did not affect their bifidogenic properties; however, structural diversity in pectic compounds is possible as long as significant arabino- and galacto-oligosaccharide content is present. Fanaro et al. (2005) investigated the effect of acidic oligosaccharides from pectin on intestinal flora and stool characteristics in infants, showing that they were well tolerated as ingredient in infant formulae but did not affect intestinal microecology.

To the best of our knowledge, the fermentation and prebiotic properties of pectin derived from artichoke (Sabater et al. 2018) and sunflower (Muñoz-Almagro et al. 2018a) by-products have not been explored. In the case of artichoke, only one previous study showed a selective growth of two specific strains, i.e. *Lactobacillus plantarum* 8114 and *Bifidobacterium bifidum* ATCC 11863 which was ascribed to the combination of its high inulin and low methylated pectin contents (Fissore et al. 2015). Also, Costabile et al. (2010) reported, in a double-blind, cross-over study carried out in healthy adults, a pronounced prebiotic effect (i.e., increasing of bidifobacteria and lactobacilli) of a very-long-chain inulin derived from artichoke on the human faecal microbiota composition. The lack of knowledge of potential alternative sources of active pectic compounds for human consumption is surprising as previous studies reported that structure and composition can make a

significant difference to the fermentation properties (Onumpai et al. 2011). Thus, bifidogenic properties seem to highly depend on the composition and structure of pectins, with neutral sugar content and GalA:Rha ratio being critical factors (Di et al. 2017).

In this context, considering the structural diversity of pectins dependent on their origin, the aim of this study was to evaluate the effect of a variety of pectins and enzymatic-modified pectins from different sources (in particular, citrus, sunflower and artichoke) on the profile changes in human faecal microbiota population and fermentation metabolites, i.e. short-chain fatty acids.

Materials and methods:

Raw material. Sunflower by-products based on heads and leftover stalks and artichoke by-products derived from external bracts, leaves and stems, were supplied by Syngenta AG and Riberebro S.L. (Spain), respectively. Prior to experiments, raw material was ground with a knife mill to particle size < 500 µm. Commercial citrus pectin (trade name Ceampectin®, ESS-4400) was kindly provided by CEAMSA (Porriño, Pontevedra, Spain).

Pectin extraction and modification. Sunflower pectin was extracted from 1 kg of dried substrate by suspending in 20 L of sodium citrate (0.7 %) at 52 ºC, pH 3.2 for 184 min under agitation and the residue was precipitated with

ethanol and then freeze-dried (Muñoz-Almagro et al. 2018a). Artichoke pectin was extracted using a cellulase from *Trichoderma reesei* (Celluclast® 1.5 L, Novozymes, Bagsvaerd, Denmark) in an orbital shaker at 50 °C, pH 5 with constant shaking (200 rpm) following the method described by Sabater et al. (2018). After hydrolysis, samples were centrifuged (1,300 x *g* for 10 min at 4 °C) and supernatants were filtered through cellulose paper. Residues were washed and precipitated in 70 % ethanol, centrifuged (1,200 x *g*, 20 min) and then freeze-dried. Extraction yield of pectin (expressed as percentage) represents the amount of pectin extracted from 100 g of initial dried raw material, being 10.0% and 22.1% the obtained values for sunflower and artichoke pectin, respectively.

The extracted sunflower and artichoke pectins, as well as the commercial citrus pectin were then subjected to an enzymatic treatment using a commercial cellulase from *Aspergillus niger* (Sigma Aldrich, Steinheim, Germany) with pectinolytic activity to reduce their Mw. Then, the resulting material was transferred to a continuous membrane reactor to separate the modified pectin from oligosaccharides and free sugars formed (Olano-Martin et al. 2001). The reactor consisted of an ultrafiltration dead-end stirred cell (model 8000, Amicon, Watford, U.K.) where the substrate was added and then pushed from a pressurized feed tank filled with water at a rate matching

the permeate flow rate. All filtrations were carried out with an Ultracel® ultrafiltration disk membrane, with a Mw cut-off (MWCO) of 3 kDa and a diameter of 76 mm as determined by the manufacturers. Checking of absence of low molecular weight carbohydrates in the ultrafiltered samples was accomplished by the analysis of the resulting retentates and permeates by SEC-ELSD following the method described in subsection 2.3.2. All pectin and MP samples were free from monosaccharides, as well as oligosaccharides below 10 kDa (**Figure 1**).

Characterisation of pectin and enzymatic-modified pectin samples.

Monosaccharide analysis. Monosaccharide analysis was performed after the acid hydrolysis of samples with 2 M trifluoroacetic acid (TFA) at 110 °C for 4 h. After that, released monosaccharides were analysed by gas chromatography (GC) carried out with an Agilent Technologies gas chromatograph (7890A) equipped with a flame ionisation detector (FID). Prior to GC analysis, trimethylsilyl oximes (TMSO) of monosaccharides were formed (Cardelle-Cobas et al. 2009b). 500 µL of hydrolysed samples were evaporated to remove the acid and then 400 µL of phenyl-β-glucoside (0.5 mg/mL) used as internal standard (I.S.) were added. Afterward, the mixture was dried at 40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland).

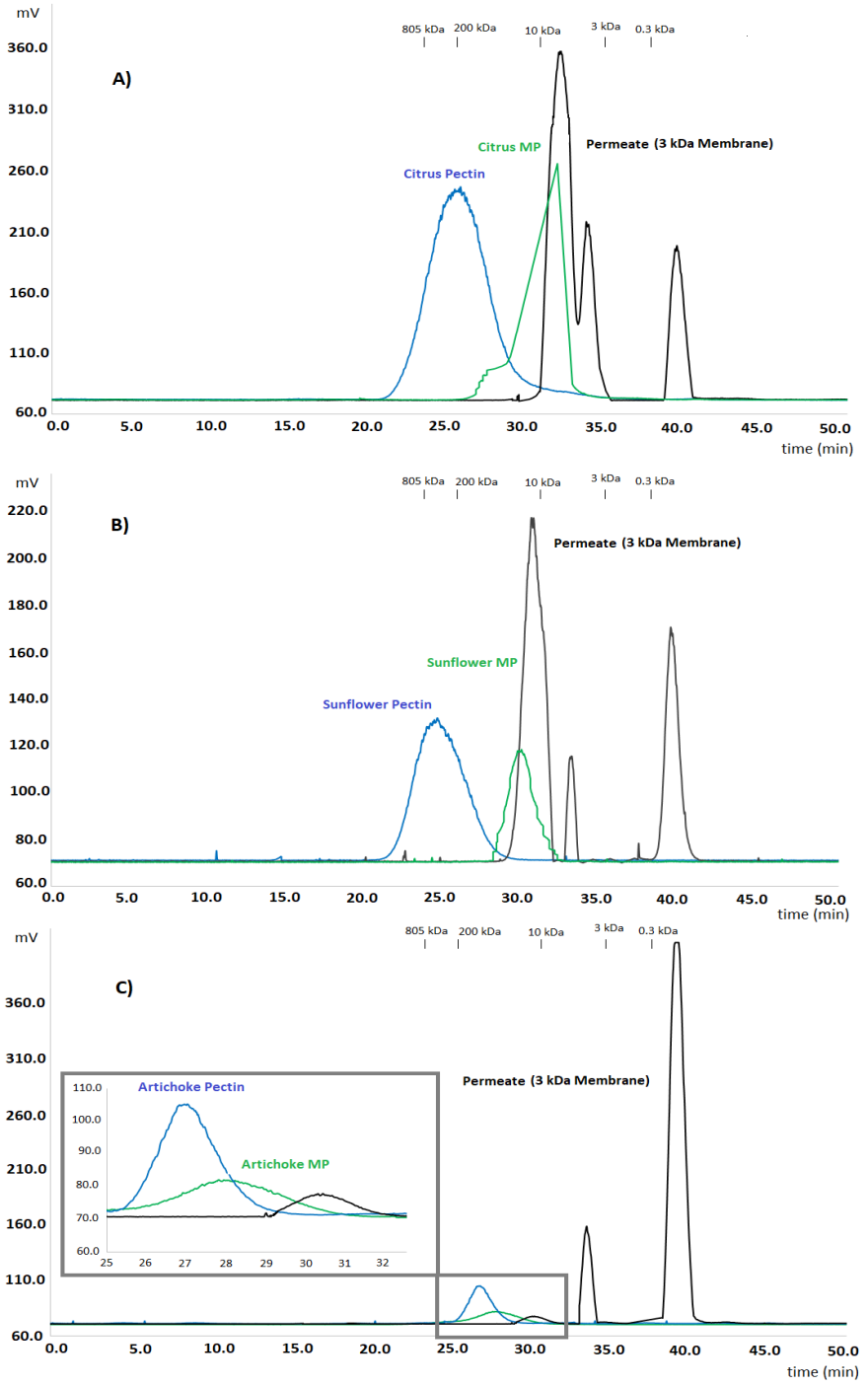

Figure. 1. SEC-ELSD profiles of pectins (blue), enzymatic-modified pectins (MP) (green), and corresponding ultrafiltrated permeates (black) derived from A) citrus, B) sunflower, and C) artichoke sources. Elution positions of standard polysaccharide polymers (pullulans) are indicated by arrows.

Sugar oximes were formed by adding 250 µL hydroxylamine chloride (2.5%) in pyridine and heating the mixture at 70 °C for 30 min. Subsequently, the oximes obtained in this step were silylated with hexamethyldisylazane (250 µL) and TFA (25 µL) at 50 °C for 30 min. Derivatisation mixtures were centrifuged at 6,700 x *g* for 2 min and supernatants were injected in the GC-FID. Analyses were carried out using a DB-5HT capillary column (15 m x 0.32 mm x 0.10 µm, J&W Scientific, Folson, California, USA). Nitrogen was used as carrier gas at a flow rate of 1 mL/min. Injector and detector temperatures were 280 and 385 °C, respectively. The oven temperature was programmed from 150 to 380 °C at a heating ratio of 1 °C/min until 165 °C and then up to 300 °C at a heating rate of 10 °C/min. Injections were made in the split mode (1:5).

Data acquisition and integration were done using Agilent ChemStations software (Wilmington, DE, USA). Response factors were calculated after duplicate analysis of standard solutions (glucose, mannose, rhamnose, arabinose, galactose, GalA and xylose) over the expected concentration range in samples, (0.01–2 mg) and IS (0.2 mg).

Estimation of the molecular weight (Mw). Estimation of Mw was carried out by Size Exclusion Chromatography (SEC) according to the method described by (Muñoz-Almagro et al. 2018b). The analysis was performed on

a LC Agilent Technologies 1220 Infinity LC System 1260 (Agilent Technologies, Germain), equipped with two consecutive TSK-GEL columns (G5000 PW_{XL}, 7.8 x 300 mm, particle size 10 µm, G2500 PW_{XL}, 7.8 x 300 mm, particle size 6 µm) connected in series with a TSK-Gel guard column (6.0mm×400mm) (Tosoh Bioscience, Stuttgart, Germany). Samples (20 µL) were eluted with 0.1 M NaCl at a flow rate of 0.5 mL/min for 50 min at 30 °C. The eluent was monitored with an Evaporative Light Scattering Detector (ELSD) (Boeblingen, Germain) at 30 °C. Pullulans of Mw 788, 473, 212, 100, 1.3, 0.34 kDa were used as standards to calibration. All the Mw values specified were weight-average.

Estimation of the degree of methoxylation. Degree of methoxylation of samples was determined by Fourier transform infrared spectroscopy (FTIR). KBr discs were prepared mixing the pectin and enzymatic-modified pectin samples with KBr (1:100) and pressing. FTIR spectra Bruker IFS66v (Bruker, US) were collected in absorbance mode in the frequency range of 400-4000 cm^{-1}, at a resolution of 4 cm^{-1} (mid infrared region) with 250 co-added scans. The degree of methoxylation was determined as the average of the ratio of the peak area at 1747 cm^{-1} (COO-R) and 1632 cm^{-1} (COO^-) as previously described (Singthong et al. 2004).

Determination of *in vitro* fermentation properties and prebiotic activities.

Faecal Inocula. Faecal samples from five healthy adults (2 male, 3 female, mean age of 30.6 ± 4.2 years old) who had not consumed prebiotic or probiotic products, nor had received antibiotic treatment within 3 months before study were obtained *in situ*. Samples were kept in an anaerobic cabinet and used within a maximum of 15 min after collection. Faecal samples were diluted (10% w/w) in anaerobic phosphate-buffered saline (PBS; 0.1 mol/L, pH 7.4, Oxoid, Basingstoke, UK) and homogenised in a stomacher (Stomacher 400, Seward, UK) at normal speed for 2 min.

***In vitro* batch fermentations.** Sterile stirred batch culture fermentation systems were set up and aseptically filled with a volume of sterile, basal medium: (per litre) 2 g peptone water, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2 \cdot 6H_2O$, 2 g $NaHCO_3$, 2 mL Tween 80, 0.05 g haemin, 10 μL vitamin K1, 0.5 g L-cysteine HCl, 0.5 g bile salts and 4 mL resazurin (0.25 g/L). Medium was sterilised at 120 °C for 30 min before aseptically dispensing into the sterile fermenters. Sterile stirred fermenters were filled with 9 mL of autoclaved basal medium and were gassed overnight by constant sparging oxygen-free nitrogen to maintain anaerobic conditions. 100 mg of substrates were added

(final concentration of 1% (w/v)) to the respective fermentation just prior to the addition of the faecal inoculum (1 mL). The temperature was maintained at 37 °C using a water jacket and the pH was maintained between 6.7 and 6.9 using an automated pH controller (Fermac 260; Electrolab, Tewkesbury, UK). The batch cultures were run for a period of 48 h and samples were taken from each vessel at 0 and 24 h for bacterial enumeration by fluorescent *in situ* hybridisation (FISH) and at 0, 10, 24, 36 and 48 h for SCFA by GC-FID. 3 extra vessels with inulin, fructooligosaccharides (FOS) and no added carbohydrate source were also included as positive and negative control, respectively.

Short-chain fatty acid (SCFA) analysis. Before chemical analysis, samples from each fermentation time were centrifuged at 13,000 x *g* for 10 min to obtain the supernatant. The clear solutions were kept at -20 °C until analysis. SCFA analysis was carried out using GC-FID based on the method described by (Richardson et al. 1989). Before analysis, samples were thawed on ice and then vortexed. After that, 400 μL of each sample were taken into a glass tube and 25 μL of 2-ethylbutyric acid (0.1 M) (IS) was added. Following that, 250 μL of concentrated HCl and 1.5 mL of diethyl ether were added and the solution was mixed 1 min and centrifuged 10 min at 2,000 x *g*. 400 μL of the upper layer (ether layer) was transferred to a GC screw-cap vial and 50 μL of

N-(*tert*-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) was added and leave 72 h to produce fully derivatisation.

A 5890 Series II Gas Chromatograph (Hewlett Packard) fitted with a Rtx-1 10 m x 0.18 mm column with a 0.20 µm coating (Crossbond 100 % dimethyl polysiloxane; Restek) was used for analysis. Helium was used as carrier gas at a flow rate of 0.7 mL/min. Injector and detector temperatures were 275 °C. Oven temperature was programmed from 63 °C for 3 min and then heated to 190 °C at a heating ratio of 3 °C/min and held at 190 °C for 3 min. Injections were made in the split mode (100:1). SCFA standards analysis was also carried out to quantify concentrations of all compounds.

Enumeration of bacterial populations. Enumeration of the target faecal bacteria groups was achieved by FISH with fluorescently labelled 16S rRNA probes according to the method described by (Wagner et al. 2003). Briefly, 375 µL aliquots were obtained from each fermenter and were mixed with 1.125 mL 4% (w/v), ice-cold paraformaldehyde and fixed for 4-10 h at 4 °C. Fixed cells were then centrifuged at 13,000 x *g* for 5 min and washed twice on 1 mL cold filter-sterilised PBS (0.1 M). The washed cells were then resuspended in 150 µL PBS and 150 µL of absolute ethanol (99 %) and stored at −20 °C until analysis.

To obtain an appropriate number of fluorescent cells in each field of view of the microscope, samples to hybridise were then diluted in a suitable volume of PBS with 1% (v/v) of sodium dodecyl sulphate, and 20 µL of the dilution was added to each well of a six-well polytetrafluoroethylene/poly-L-lysine-coated slide (Tekdon Inc., Myakka City, USA). Samples were dried at 48-50 °C for 15 min in a desktop plate incubator and dehydrated in an alcohol series (50, 80 and 96% (v/v) ethanol, 2 min each) and placed again at 48-50 °C to evaporate the excess of ethanol before adding the hybridisation solution. 50 µL of hybridisation solution (per 1 mL; 5 M NaCl 180 µL, 1 M Tris/HCl 20 µL, ddH_2O 799 µL, 1 µL SDS 10% (w/v) and 100 µL of probe) was added to each well and left to hybridise for 4 h in a microarray hybridisation incubator (Grant-Boekel, UK) at 46-50 °C depending on the probe. After hybridisation, slides were washed in 50 mL washing buffer (5 M NaCl 9 mL, ddH_2O 40 mL and 1 M Tris/HCl 1 mL) for 15 min and dipped in cold distilled water for 2-3 seconds. Slides were then dried with compressed N_2 and a drop of PVA-DABCO antifade (polyvinyl alcohol mounting medium with 1,4-diazabicyclo (2.2.2) octane) was added onto each well. A coverslip (20 mm, thickness no. 1; VWR) was placed on each slide and cell numbers of microorganisms were determined by direct counting under an epifluorescence microscope (Eclipse 400; Nikon, Surrey, UK) with Fluor 100 lens. A total of 15 random fields of view were counted for each well.

The oligonucleotide probes used and conditions for each one are detailed in **Table 1**. These probes were selected to account for major bacterial groups in the Actinobacteria (Bif164), Bacteroidetes (Bac303), and Firmicutes (Lab158, Erec482, Chis150) phyla.

Table 1. Oligonucleotide probes used in this study for FISH enumeration of bacteria.

Probe	Specificity	DNA Sequence (5' to 3')	Temperature (°C)		Reference
			HB*	WB*	
Bac303	Most *Bacteroidaceae* and *Prevotellaceae*. some *Porphyromonadaceae*	CCA ATG TGG GGG ACC TT	46	48	Manz et al. (1996)
Bif164	*Bifidobacterium spp.*	CAT CCG GCATTA CCA CCC	50	50	Langendijk et al. (1995)
Chis150	Most of the *Clostridium histolyticum* group (Clostridium cluster I and II)	TTA TGC GGT ATT AAT CT(C/T) CCT TT	50	50	Franks et al. (1998)
Erec482	Most of the *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa and XIVb)	GCTTCT TAGTCA (A/G)GT ACC G	50	50	Franks et al. (1998)
Lab158	*Lactobacillus; Enterococcus*	GGT ATT AGC A(C/T)C TGT TTC CA	50	50	Harmsen et al. (1999)

*HB: hybridisation buffer; WB: washing buffer

Statistical analysis. Statistical analysis was performed using SPSS for Windows, version 23.0. One-way analysis of variance (ANOVA) and Tukey's *post hoc* test was used to determine significant differences among the bacterial group populations and organic acid concentrations among the

different substrates. Differences were considered significant at $p < 0.05$ (n=5).

Results and discussion:

The yields of extraction of pectin from artichoke (22.1%) and sunflower by-products (10.0%) were in line with those obtained for other well-established sources of pectin, such as citrus peel (Kurita et al. 2008), lime peel (Dominiak et al. 2014), apple pomace (Wikiera et al. 2015) or passion fruit peels (Liew et al. 2016), suggesting their potential use as renewable pectin sources.

Characterisation of pectin and enzymatic-modified pectin samples. Pectins from different sources (that is, citrus, artichoke and sunflower) and their enzymatic modified polysaccharides (modified pectin (MP)) were evaluated in this study. Neutral sugars and GalA content, average degree of methoxylation and average estimated Mw are included in **Table 2**. The GalA:Rha ratio displayed in the table shows the number of GalA residues per Rha residue, giving an indication of the RG-I backbone respect to HG content. Thus, a lower value shows a compound richer in RG-I chains. Ara:Rha and Gal:Rha ratios indicate the number of neutral sugar residues attached to the RG-I backbone.

As expected, GalA was the major monosaccharide residue in all pectic samples, ranging from 46.5 % (w/w) to 88.1% (w/w). The lowest values of GalA content were observed in those samples which had the highest values of rhamnose content. In consequence, the GalA:Rha ratio indicated that citrus MP, artichoke pectin, artichoke MP and citrus pectin were the most enriched samples in RG-I as compared to sunflower samples, which were the most enriched in HG structure according to the monomeric composition (27.4 and 24.1 for GalA:Rha ratio for sunflower pectin and sunflower MP, respectively). Instead, artichoke pectin and MP presented high amounts of arabinose, surpassing rhamnose content, which could be indicative of a highly enriched structure in arabinan and arabinogalactan branches to the RG-I chains. The amount of rhamnose and arabinose with respect to GalA may also indicate the substitution of the rhamnogalacturonan branching along the HG with arabinan and arabinogalactan structures (Manderson et al., 2005; Yuliarti et al. 2015). The high content of arabinose and GalA determined in artichoke samples support the data obtained in previous studies (Femenia et al. 1998; Sabater et al. 2018). Galactose content in all samples was higher than other neutral sugars, with the exception of arabinose in artichoke pectin, which may also indicate the presence of galactose-based oligosaccharides branched to the HG backbone.

Table 2. Chemical characterisation of pectins and enzymatic-modified pectins from different renewable bioresources**.**

Sample	Monosaccharide (%*)							Average Mw (kDa)	GalA:Rha	Ara:Rha	Gal:Rha	Average degree of methoxylation (%)
	Xylose	Arabinose	Rhamnose	Galactose	Mannose	Glucose	Galacturonic acid					
Citrus Pectin	0.9 ± 0.0	3.5 ± 0.0	5.8 ± 0.0	20.2 ± 0.1	1.4 ± 0.0	1.8 ± 0.0	66.5 ± 0.2	800-100	11.52	0.61	3.50	70.7
Citrus MP	1.3 ± 0.2	3.7 ± 0.2	9.8 ± 0.1	14.0 ± 0.3	2.4 ± 0.3	13.3 ± 0.3	55.6 ± 0.6	12.0-10.0	5.70	0.38	1.44	14.2
Sunflower Pectin	2.2 ± 0.1	1.1 ± 0.0	3.2 ± 0.4	4.3 ± 0.0	0.1 ± 0.0	0.9 ± 0.0	88.1 ± 0.9	800-100	27.39	0.35	1.35	45.7
Sunflower MP	0.9 ± 0.0	2.3 ± 0.0	3.2 ± 0.1	12.2 ± 0.0	1.3 ± 0.0	1.8 ± 0.0	78.2 ± 0.5	12.5	24.13	0.71	3.77	17.0
Artichoke Pectin	1.1 ± 0.1	18.9 ± 0.6	7.6 ± 0.1	8.2 ± 0.3	1.0 ± 0.3	16.7 ± 0.7	46.5 ± 0.6	>500	6.13	2.50	1.09	8.9
Artichoke MP	2.3 ± 0.2	10.7 ± 0.1	5.4 ± 0.0	21.1 ± 0.0	1.2 ± 0.0	3.9 ± 0.0	55.5 ± 0.8	300-80	10.34	1.99	3.94	8.5

Analysis were carried out at least in duplicate (n=2)
*Monosaccharide content (%) is referred regarding the total carbohydrate measured on each sample.

Xylose that can be present in more complex structural features of pectin, such as RG-II regions or arabinoxylans and xylogalacturonan (Maxwell et al. 2012), ranged from 0.9% to 2.3%. Lastly, glucose (from 0.9% to 16.7%) and mannose (from 0.1% to 2.4%) were found in all samples and they could likely derive from non-pectic polysaccharides extracted in minor amounts together the target pectins, such as xyloglucan, hemicellulose, and/or cellulose (Yapo 2009; Wang et al. 2016; Sabater et al. 2018).

In both artichoke samples the degree of methoxylation was the lowest (8.9 and 8.5 % for pectin and MP, respectively), whereas MP samples from citrus and sunflower had moderately higher values (14.2 and 17.0 %, respectively) and citrus and sunflower pectin had the highest data of all samples with 70.7 % and 45.7 % of degree of methoxylation, respectively. This behaviour could be ascribed to the pectin methyl esterase activity of the enzyme employed to produce the corresponding MP.

On the other hand, all resulting MP showed a reduction of the Mw as compared to their respective pectin due to the polygalacturonase enzyme activity, which was concomitant with a decrease in GalA and an increase in RG-I to HG. However, modified artichoke pectin showed a decrease in arabinose which led to a higher relative content of GalA compared to its parent pectin. The initial high content of arabinose observed in artichoke

pectin could be related to the resulting high Mw of artichoke MP following enzymatic treatment. It is well known that arabinose is present in pectin as arabinan side chains and, consequently, a high degree of branching may create steric hindrance impairing the efficient cutting of the main chain composed by GalA. The decrease in Mw was correlated to the diminution of degree of methoxylation observed in citrus and sunflower samples. It is interesting to note that citrus and sunflower MP exhibited a Mw of 10-12.5 kDa which is in line with other modified pectins obtained from citrus (~ 10 kDa) that have shown to be effective supplements in the treatment of cancer and other diseases (Morris et al. 2013). Artichoke MP showed a small decrease in this parameter which is in accordance with its high Mw, as shown in **Figure 1**.

***In vitro* fermentation.**

Bacterial population changes during *in vitro* fermentation. Changes in the human faecal bacterial populations during the *in vitro* fermentation with the different pectins and enzymatic-modified pectins after 24 h are shown in **Table 3**. A significant increase ($p < 0.05$) of *Bifidobacterium* (Bif164) population for all carbohydrate samples was observed after 24 h of fermentation. It is well known that oligosaccharides deriving from pectins have bifidogenic activities, however there are also studies that have

demonstrated a bifidogenic effect in intact pectins suggesting a potential role of this polysaccharide as a prebiotic (Gómez et al. 2016; Yang et al. 2013). In our study, numerical increases up to 0.79 – 1.19 $\log_{10}$ in population were determined. Some authors indicated that increments of 0.5 - 1.0 $\log_{10}$ in bifidobacteria could be considered as a major shift in the gut microbiota towards a potentially healthier composition of intestinal microbiota (Kolida & Gibson 2007). Thus, all pectic samples could be considered bifidogenic under the studied conditions. Remarkably, artichoke MP was the substrate, which promoted the significantly highest growth in bifidobacteria among all assayed samples, including positive controls as inulin and FOS which in turn showed a similar bifidobacterial growth as compared to sunflower and citrus samples. This fact could be attributed to the high combined content of arabinose and galactose found in artichoke MP (**Table 2**) according to previous studies reporting a correlation between arabinose and galactose content with bifidogenic properties (Di et al. 2017; Manderson et al. 2005; Onumpai et al. 2011). Moreover, a positive effect of the decrease of Mw in pectin on its ability to promote bifidobacteria growth was observed for citrus and artichoke sources since their MP derivatives exhibited a significant ($p < 0.05$) increase as compared to unmodified pectin (9.63 vs 9.42 $\log_{10}$ for citrus and 9.82 vs. 9.50 for artichoke), whereas sunflower pectin and MP presented a statistically identical bifidogenic activity.

Table 3. Bacterial populations (log10 cells per ml) enumerated by FISH at 0 and 24 h of in vitro fermentation with Inulin, FOS, citrus pectin, citrus modified pectin (MP), sunflower pectin, sunflower MP, artichoke pectin and artichoke MP.

Probe/Strain	Time point (h)	Bacterial concentration (log10 cells/mL)								
		Control	Inulin	FOS	Citrus Pectin 800-100 kDa	Citrus MP 10.0 – 12.0 kDa	Sunflower Pectin 800-100 kDa	Sunflower MP 12.50 kDa	Artichoke Pectin > 500 kDa	Artichoke MP 300-80 kDa
Bif164	**0**	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)
	24	8.75 (0.03)^{a}	9.52 (0.15)^{bc,1}	9.48 (0.05)^{bc,1}	9.42 (0.06)^{b,1}	9.63 (0.04)^{cd,1}	9.72 (0.12)^{cd,1}	9.74 (0.06)^{cd,1}	9.50 (0.14)^{bc,1}	9.82 (0.13)^{d,1}
Bac303	**0**	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)
	24	8.59 (0.08)^{a}	9.36 (0.05)^{ef,1}	9.39 (0.04)^{f,1}	9.05 (0.08)^{bc,1}	9.06 (0.03)^{bc,1}	9.02 (0.09)^{b,1}	9.19 (0.07)^{cd,1}	9.23 (0.11)^{de,1}	9.45 (0.04)^{f,1}
Lab158	**0**	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)
	24	8.38 (0.07)^{a}	9.05 (0.04)^{de,1}	8.98 (0.03)^{cd,1}	8.65 (0.06)^{b,1}	9.04 (0.03)^{d,1}	9.05 (0.02)^{d,1}	8.98 (0.09)^{cd,1}	8.92 (0.05)^{c,1}	9.17 (0.05)^{e,1}
Erec482	**0**	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)
	24	8.51 (0.04)^{a}	8.97 (0.11)^{bc,1}	9.08 (0.11)^{c,1}	9.02 (0.05)^{bc,1}	9.06 (0.06)^{c,1}	8.83 (0.11)^{b,1}	8.97 (0.07)^{bc,1}	8.95 (0.06)^{bc,1}	9.01 (0.11)^{bc,1}
Chis150	**0**	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)
	24	8.35 (0.04)^{a}	8.77 (0.06)^{b,1}	8.72 (0.03)^{b,1}	8.70 (0.09)^{b,1}	8.73 (0.03)^{b,1}	8.77 (0.01)^{b,1}	8.70 (0.02)^{b,1}	8.72 (0.04)^{b,1}	8.70 (0.06)^{b,1}

A control sample without carbohydrate source is also included. Experiments were carried out in batch cultures systems inoculated with faecal inocula from five healthy human donors. Results shown as mean (n = 5) with the corresponding standard deviation in parentheses.

[a. b. c] Significant differences ($p < 0.05$) between substrates are indicated with different letters in the same row.

[1] Significant difference ($p < 0.05$) from the 0 h value for each bacterial group and for the same substrate.

Evidently, there was not any significant increase during fermentation of negative controls, confirming the suitability of these substrates as a carbon source for the metabolism of bifidobacteria. The degree of methoxylation did not have impact on the bifidogenic properties. More specifically, sunflower samples had different value of this parameter with the same bifidogenic activity and artichoke samples had almost the same one with different bifidogenic activity.

The second highest increase (up to 0.56 – 0.93 $\log_{10}$) was observed in *Bacteroides/Prevotella* (Bac303) population. This general increase is explained by the fact that *Bacteroides* species are major carbohydrate-degrading organisms in the gut and have the capacity to degrade diverse plant polysaccharides, including pectins (Dongowski et al. 2000; Flint et al. 2012; Onumpai et al. 2011). Indeed, many *Bacteroides* strains from human faeces can produce pectinolytic enzymes, including polygalacturonase and pectin methylesterase (Dekker & Palmer 1981; Jensen & Canale-parola 1986). Therefore, *Bacteroides* can be involved in cross-feeding with *Bifidobacteria* by releasing breakdown products of pectin or MP which might be utilised by the latter, thus, promoting their growth. Inulin, FOS and artichoke MP samples exhibited the highest increase in *Bacteroides*. With respect to the effect of Mw on *Bacteroides/Prevotella* growth, sunflower and artichoke MP

demonstrated a significantly higher increase than their respective pectins. This difference could be attributed to the galactan chains branched to the RG-I since Gal:Rha ratio increased in both sunflower and artichoke MP after the enzymatic hydrolysis.

A significant increase in *Lactobacillus/Enterococcus* (Lab158) was also observed for all tested carbohydrate samples, with the most significant increases found in inulin and artichoke MP. Similar to *Bifidobacterium*, *Lactobacillus* is considered one of the major microbial targets for prebiotic action due to their health effects. The high increment in *Lactobacillus/Enterococcus* population following artichoke MP fermentation further established the correlation of arabinose and galactose content with the prebiotic properties. Mw did not affect sunflower samples but it seemed to have an impact on citrus and artichoke sources, in a similar manner to the behaviour observed for *Bifidobacterium* selectivity.

Clostridium coccoides/Eubacterium rectale (Erec482) showed a significant increase in all tested samples but no significant differences were found among any of the carbohydrate substrates including inulin and FOS. Increase in *Eubacterium rectale* is of particular interest due to its ability to produce butyrate (Manderson et al. 2005). Di et al. (2017) reported an increase of Erec482 numbers when testing a citrus MP of similar Mw (9.2 kDa),

although they did not find a positive correlation with the determined butyrate concentrations. In the same way, Chen et al. (2013) reported enhanced *Eubacteria* growth on apple pectin compared to the respective POS, suggesting that the Mw was not a relevant factor. In our work, similar behaviour was observed since all pectic samples resulted in a significant stimulation of the butyrate producing bacteria groups (Erec482) and no differences were found between samples with different Mw or origin.

Clostridium histolyticum (Chis150) population displayed the lowest changes in all cases, leading to a rather moderate increase (lower than 0.5 $\log_{10}$) after 24 h of fermentation. No significant differences among any substrates were observed after fermentation. In general, *Clostridium* species are considered as potentially harmful bacteria, so in this way, all pectic samples induced a favourable behaviour.

Short-chain fatty acids (SCFA) production. Acetate, propionate, butyrate and total SCFA formation was analysed throughout the fermentation in batch cultures (**Table 4**). Total SCFA concentration increased strongly during the first 10 or 24 h of fermentation in all tested substrates. In general terms, neither the degree of methoxylation nor Mw of pectin samples had an influence on the SCFA production, as reflected by the values contained in **Table 4**.

Table 4. SCFA concentrations (mM) determined by GC-FID at 0, 10, 24, 36 and 48 h on *in vitro* fermentations with Inulin, FOS, citrus pectin, citrus modified pectin (MP), sunflower pectin, sunflower MP, artichoke pectin and artichoke MP.

SCFA	Time point (h)	Mean SCFA concentration (mM) in substrate								
		Control	Inulin	FOS	Citrus Pectin 800-100 kDa	Citrus MP 10.0 – 12.0 kDa	Sunflower Pectin 800-100 kDa	Sunflower MP 12.50 kDa	Artichoke Pectin > 500 kDa	Artichoke MP 300-80 kDa
Acetate	0	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)
	10	12.28 (3.30)^{a,1}	36.99 (6.60)^{b,1}	54.64 (15.10)^{bcd,1}	61.65 (11.81)^{cd,1}	68.49 (6.19)^{d,1}	50.64 (9.15)^{bcd,1}	58.47 (4.13)^{cd,1}	49.35 (5.54)^{bcd,1}	42.19 (3.77)^{bc,1}
	24	21.11 (3.31)^{a,2}	62.44 (11.68)^{bcd,2}	57.89 (14.88)^{bc}	78.42 (9.02)^{cd}	78.83 (12.87)^{cd}	82.65 (11.80)^{d,2}	69.21 (10.29)^{bcd}	55.33 (1.62)^{b}	50.86 (7.81)^{b}
	36	26.18 (4.49)^{a}	65.24 (11.98)^{bc}	63.68 (10.80)^{bc}	71.18 (11.38)^{bc}	78.95 (11.71)^{c}	78.95 (11.62)^{c}	67.64 (9.27)^{bc}	55.64 (4.57)^{b}	55.60 (11.09)^{b}
	48	17.62 (3.38)^{a}	64.42 (10.55)^{bc}	63.55 (10.86)^{bc}	77.99 (14.69)^{bc}	85.40 (11.34)^{c}	78.49 (13.31)^{bc}	73.87 (10.49)^{bc}	61.00 (12.27)^{bc}	55.94 (8.95)^{b}
Propionate	0	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)
	10	2.28 (0.92)^{a}	7.58 (1.91)^{ab}	12.20 (6.90)^{b}	8.13 (3.22)^{ab,1}	11.6 (3.76)^{b,1}	6.35 (2.33)^{ab,1}	10.00 (1.10)^{ab,1}	11.35 (4.10)^{b,1}	11.27 (3.17)^{b,1}
	24	4.7 (1.26)^{a,2}	18.17 (4.69)^{b,2}	15.64 (6.76)^{b,1}	12.12 (4.58)^{ab}	16.04 (1.86)^{b}	11.82 (1.26)^{ab,2}	13.84 (0.90)^{b,2}	13.49 (3.98)^{b}	15.69 (1.37)^{b,2}
	36	4.1 (0.60)^{a}	19.51 (5.68)^{cd}	23.77 (2.89)^{d}	11.92 (4.26)^{b}	16.15 (2.26)^{bcd}	11.80 (1.47)^{b}	14.12 (1.61)^{bc}	14.55 (3.51)^{bc}	16.97 (1.62)^{bcd}
	48	2.12 (0.99)^{a}	18.41 (4.95)^{bc}	20.69 (6.22)^{c}	12.71 (3.51)^{b}	17.35 (2.12)^{bc,2}	13.60 (3.01)^{bc}	14.15 (1.82)^{bc}	14.10 (3.61)^{bc}	16.10 (2.03)^{bc}
Butyrate	0	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)
	10	1.29 (0.64)^{a}	6.57 (0.75)^{b}	3.43 (2.17)^{ab}	1.82 (1.94)^{a}	2.51 (1.36)^{a}	2.22 (1.10)^{a}	3.26 (1.52)^{ab}	2.70 (1.88)^{a}	2.74 (1.12)^{a}
	24	1.77 (1.03)^{a}	9.13 (2.22)^{b,1}	5.20 (3.28)^{ab}	4.52 (1.94)^{a,1}	4.94 (1.54)^{ab,1}	4.54 (2.19)^{a,1}	5.42 (1.59)^{ab,1}	5.30 (1.68)^{ab,1}	4.50 (0.78)^{a,1}
	36	2.31 (0.67)^{a,1}	9.66 (3.14)^{c}	7.60 (3.20)^{bc,1}	5.86 (2.26)^{abc,2}	5.35 (1.87)^{abc,2}	5.13 (1.88)^{abc}	6.23 (1.80)^{abc}	5.25 (2.22)^{abc}	4.98 (1.34)^{ab}
	48	1.06 (0.38)^{a}	9.08 (2.87)^{b}	8.40 (3.85)^{b}	4.92 (2.08)^{ab}	5.89 (1.59)^{ab}	4.78 (1.31)^{ab}	6.33 (2.61)^{b}	6.04 (3.04)^{b}	4.98 (1.87)^{ab}
Total	0	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)
	10	15.84 (4.61)^{a,1}	49.14 (13.14)^{b,1}	83.76 (17.01)^{c,1}	71.42 (12.18)^{bc,1}	82.95 (6.94)^{c,1}	59.21 (11.65)^{bc,1}	71.74 (6.46)^{bc,1}	63.89 (10.69)^{bc,1}	56.20 (7.58)^{b,1}
	24	27.83 (3.69)^{a,2}	90.30 (11.28)^{bcd,2}	84.14 (17.36)^{bcd}	94.15 (11.21)^{bcd}	102.36 (14.94)^{d}	99.01 (11.79)^{cd,2}	89.70 (9.42)^{bcd}	74.99 (3.97)^{bc}	72.42 (9.24)^{b}
	36	32.24 (4.55)^{a}	95.90 (13.77)^{b}	90.74 (15.10)^{b}	88.97 (14.71)^{b}	97.03 (18.08)^{b}	95.88 (12.16)^{b}	89.64 (10.97)^{b}	77.27 (8.63)^{b}	79.42 (13.64)^{b,2}
	48	21.21 (3.96)^{a}	90.38 (18.27)^{bc}	91.45 (11.89)^{bc}	95.63 (16.72)^{bc}	109.42 (12.10)^{c}	96.87 (13.57)^{bc}	98.84 (9.49)^{bc,2}	83.19 (17.16)^{bc}	77.02 (11.35)^{b}

A control sample without carbohydrate source was also included. Experiments were carried out in batch cultures systems inoculated with faecal inocula from five healthy human donors. Results shown as mean (n = 5) with the corresponding standard deviation in parentheses.

[a. b. c] Significant differences ($p < 0.05$) between substrates are indicated with different letters in the same row.

Acetate was the most abundant SCFA, followed by propionic and butyric acids in all substrates. Formation of acetate has been related to an enhancement of the ileal motility, a protection against genotoxic agents and pathogens and an increase of colonic blood (Hong et al. 2005). In our study, the only significant differences found between samples after 48 h of analysis were with artichoke and citrus MPs. Results demonstrated a sharp increase of this compound in the first 10 h of fermentation. Although it is challenging to attribute a particular fermentation end-product to a specific bacterial group in a mixed culture system, overall the increase in acetate is in agreement with the dynamics of the microbial populations, since all samples promoted the growth of *Bifidobacterium* and *Lactobacillus* (**Table 3**), which are acetate producers. Additionally, these end-products may serve as substrates for other bacteria due to metabolic cross-feeding (Belenguer et al. 2006). Acetate is generated by many bacterial groups that inhabit the colon, with approximately one-third of the product coming from reductive acetogenesis (Miller & Wolin, 1996). In contrast, bacterial groups that form propionate and butyrate are specialised and are of particular interest in terms of their beneficial effects. The main propionate-producing bacteria in the human colon are *Bacteroides* and *Clostridium* whereas butyrate production is related to bacterial groups such as *Clostridium histolyticum* (clusters I, II, IV, XIVa, XV and XVI) and *Eubacterium rectale*.

An increase in propionate concentration was seen in all samples after 48 h of fermentation, whereas fermentation of inulin and FOS resulted in the highest increase among all samples. Similarly to acetate, the high variability found among the five donors meant that propionate differences between all samples were not considered statistically significant ($p > 0.05$) during the first 24 h of fermentation. However, the increase in this end-product is in good agreement with the increase in *Bacteroides* population displayed in **Table 3.** Propionate has also been shown to exert beneficial effects on host health, such as reduction of food intake and enhancement of satiety via augmentation of the satiety hormone leptin (Zeng 2014), and a protective role against carcinogenesis through the decrease in human colon cancer cell growth via hyperacetylation of histone proteins and stimulation of apoptosis (Hinnebusch et al. 2002; Jan et al. 2002).

Butyrate production resulted in a significant increase in all samples after 24 h of fermentation. FOS and inulin showed the highest increase after 48 h of fermentation, although non-significant differences were observed among all substrates due to the high inter-individual variability (**Table 4**). The low but significant increase in butyrate levels are in accordance with the increase of Erec482 and Chis150 numbers which also include some of the major butyrate-produces (*Eubacterium rectale* and *Clostridium histolyticum)*.

Although acetate, propionate and butyrate are all metabolised to some extent by the epithelium to provide energy, butyrate plays a critical role in maintaining colonic health and moderating cell growth (Zeng 2014). Compared to acetate and propionate, butyrate exhibits strong anti-inflammatory properties, likely mediated by inhibition of TNF-α production, NF-κB activation, and IL-8, -10, -12 expression in immune and colonic epithelial cells and a protective role against colon cancer (Bailón et al. 2010; Zeng 2014).

Conclusions:

Findings in this work highlight the suitability of artichoke and sunflower by-products as renewable sources of bioactive pectic compounds since the reported yields were within the range observed for other well-established pectin sources. To the best of our knowledge, this is the first evidence of prebiotic potential of pectic compounds from sunflower and artichoke and also supports the important role played by the arabinose-rich rhamnogalacturonic acids in stimulating *Bifidobacteria*. A positive effect of decreasing molecular weight on fermentation properties was found in artichoke and citrus sources since their respective enzymatically-modified pectins promoted significantly higher growth in *Bifidobacterium* and *Lactobacillus* than the corresponding unmodified pectin. In the case of

sunflower, this behaviour was only observed in *Bacteroides/Prevotella*, which also grew to significantly higher population levels on artichoke MP as compared to the unmodified pectin. No significant effects of the molecular weight of pectin samples on SCFA production were observed, although this could be due to the high inter-individual variability observed in acetate, propionate and butyrate formation. Likewise, the degree of methoxylation did not have any significant impact on the fermentability nor SCFA production, regardless the origin of the pectic compounds.

To conclude, although further *in vivo* studies should be conducted, our data reveal that either pectin or enzymatically-modified pectin from sunflower and artichoke by-products might be considered as efficient prebiotic candidates for human consumption showing similar ability to promote the *in vitro* growth of beneficial gut bacteria as *Bifidobacterium* and *Lactobacillus* in comparison to well-recognized prebiotics as inulin and FOS.

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Declarations of interest: none

Chapter 5

Behaviour of citrus pectin during its gastrointestinal digestion and fermentation in a dynamic simulator (simgi®)

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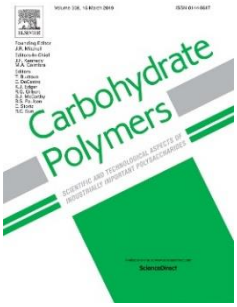

Abstract:

The behaviour of citrus pectin during digestion and its potential prebiotic properties were examined using a Dynamic Gastrointestinal Simulator (simgi®) model for the human gut, which simulates processes in the stomach, small intestine, ascending, transverse and descending colon. A remarkable non-digestibility of pectin in the upper gastrointestinal tract was observed by HPLC-ELSD analysis, where ~88% of citrus pectin remained intact during its transit through the stomach and small intestine. Fermentation of pectin stimulated the growth of beneficial bacteria such as Bifidobacterium spp, Bacteroides spp and Faecalobacterium prausnitzii. High increases of short-chain fatty acids (SCFA) were observed, especially in acetate and butyrate produced due to direct fermentation of pectin or by cross-feeding interaction between bacteria. This is the first study on the digestibility and fermentation of pectin carried out in a complex dynamic gastrointestinal simulator, being of special relevance the results obtained for *F. prausnitzii.*

Introduction:

Pectins are a family of plant cell wall polysaccharides with glycan domains that contain galacturonic acid (GalA) units with α-1,4 linkages. It mainly consists of a GalA -rich backbone, known as homogalacturonan (HG ≈ 65%), of which a number of residues are methyl esterified at the C-6 position, thereby conferring a specific degree of methoxyl esterification (DM) to the polymer. This degree of esterification and its distribution pattern define the charge distribution over the polymer playing a major role in the dimerization of pectin chains through the formation of junction zones, either via cooperative Ca^{2+} complexation or at reduced water activity as well as pH, thus defining the gelation properties of pectin (Dongowski et al. 2002; Fraeye et al. 2010). Furthermore, rhamnose residues interrupt the HG structure to form rhamnogalacturonan I (RG-I ≈ 20-35%) which is based on a backbone consisting of a repeating disaccharide of [→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→] residues. RG-I has a number of side chains attached to its backbone and the length of these chains can vary from single glycosyl to polymeric side chains of different types (1→5)-α-L-arabinans, (1→4)-β-D-galactans, arabinogalactans-I, arabinogalactans-II (Buffetto et al. 2015). RG-II constitutes ≈ 2-10% of pectin and is the most complex part of pectin, it has a HG backbone branched with rhamnose and other minor sugars such as

fucose, glucuronic acid, methyl-esterified glucuronic acid, apiose, 2-O-methylxylose, and 2-O-methylfucose (Holck et al. 2014; Lara-Espinoza et al. 2018; Noreen et al., 2017). The suitability of pectin for specific applications is governed by the structural features, including molecular weight (Mw), neutral sugar content, proportion of HG:RG-I regions or the DM (Ferreira-Lazarte et al. 2018; Sila et al. 2009). These factors can affect its applicability as thickeners or as gelling and stabilizing agents (Gullón et al. 2013).

Pectins, as other dietary fibres, are believed to be resilient to digestion reaching the hindgut where they are fermented by the colonic microbiota (Lunn & Buttriss, 2007). However, before they reach the colon, these heteropolysaccharides are subjected to the singular luminal environment of the upper digestive tract that can contribute to chemical and physicochemical changes affecting the rate and extent of the fermentation in the colon (Hoebler et al. 1998). The intestinal degradation of pectin has been studied with substantially dissimilar results. In studies involving human subjects, Chinda et al. (2003) and Saito et al. (2005) as well as Holloway (1983) observed that around 90% and 60-85% of apple and citrus pectin, respectively, reached the terminal ileum but the procedures to evaluate total pectin were not robust enough to identify the possible structural and physical changes that take place.

However, the capability of pectins to be fermented by the intestinal

microbiota it is well known, being the arabino- and galacto-oligosaccharides content one of the most important factors, even more relevant than Mw (Di et al. 2017; Onumpai et al. 2011). In this sense, there are some investigations that report a better bifidogenic effect, which means a growth of *Bifidobacteria* population, of pectins and pectic-polysaccharides with higher arabino- and galacto-oligosaccharides content, over modified pectin and pectic-oligosaccharides (POS) with lower Mw (Di et al. 2017; Ferreira-Lazarte et al. 2018).

Most of these studies are *in vitro* and often restricted to faecal samples, since *in vivo* investigations with animals and human trials have various drawbacks, such as high costs, ethical constraints, inter-individual variations and limitations in sampling from the small and large bowel (Venema & Van Den Abbeele, 2013; Verhoeckx et al. 2015). Nonetheless, even if they have limitations based on the absence of a physiological host environment, *in vitro* models are reproducible, since they allow better control of the experimental variables than animal or human studies. In general, they are rapid and simple methods and, therefore, relatively inexpensive and cost-effective. Furthermore, they allow a reduction of the samples size when this is a limiting factor (Verhoeckx et al. 2015).

Therefore, several *in vitro* models have been developed to simulate the multistage processes of human gastrointestinal digestion (Alminger et al.

2014; Cascone et al. 2016; Hur et al. 2011; Marzorati et al. 2011; Verhoeckx et al. 2015). Among all these models, complex multi-compartmental continuous systems overcome the limitations present on static models, which do not reproduce the dynamic environment of the GIT (e.g. pH changes, peristaltic movements, gastric emptying, continuous changes, and secretion flow rates) (Ouwehand & Vaughan 2006). Nowadays, dynamic gastrointestinal digestion simulators are still limited. The SIMulator Gastro-Intestinal (simgi®, Madrid, Spain) (Barroso et al. 2015) comprise five different compartments system, which simulates the different regions of the GIT such as, stomach (ST), small intestine (SI) and three compartments simulating the ascending (AC), transverse (TC) and descending (DC) regions of the human colon. The simgi® represents a fully computer controlled multi-compartmental system, which allows joint or separated simulation of the gastric and colonic fermentative processes. Thus, this is a flexible modulating system that combines a gastric compartment that simulates peristaltic mixing movements, a reactor that simulates the small intestine and three-stage continuous reactors for reproducing the colon region-specific microbiota and its metabolism (Barroso et al. 2015).

Therefore, the aim of the present study was to examine the *in vitro* gastrointestinal digestion of a commercial citrus pectin using the Dynamic

Gastrointestinal Simulator (simgi®), and its impact on the subsequent fermentation by the colonic microbiota.

Materials and methods:

Samples of pectin. Commercial citrus pectin (trade name Ceampectin®, ESS-4400) was kindly provided by CEAMSA (Porriño, Pontevedra, Spain). Galacturonic acid (GalA) content, degree of methoxylation (DM), molecular weight (Mw) and neutral sugar content of the tested pectin were described in a previous study in our laboratory (Muñoz-Labrador et al. 2018) (**Table S1, *Annex C***).

Simgi® model assays digestion. The dynamic gastrointestinal simulator simgi® was used in the operating mode to work with the five units simulating the stomach (ST), small intestine (SI) and the ascending (AC), transverse (TC) and descending colon (DC) regions (Barroso et al. 2015). **Figure 1** shows the experimental protocol of the simgi® trial. The operation of the dynamic model was validated and optimized in previous studies (Barroso et al. 2016, Barroso et al. 2015; Cueva et al. 2015). Faecal slurry was obtained from a healthy volunteer who had no received any antibiotic treatment in the previous 3 months of the experiment. Then, faecal samples were diluted (20% w/w) in sterilised phosphate-buffered saline (PBS; 0.1 mol/L, pH 7.0,

Oxoid, Basingstoke, UK) containing 1 g/L sodium thioglycolate as reduced agent. The nutritive medium was adapted from the studies mentioned before and it was constituted by potato starch (Difco™, BD) (7 g/L), glucose (Difco™, BD) (0.4 g/L), yeast extract (Oxoid, ThermoFisher Scientific) (3 g/L), special peptone (Oxoid, ThermoFisher Scientific) (1 g/L), mucin from porcine stomach (Sigma-Aldrich, Merk) (4 g/L) and L-cysteine (Panreac AppliChem) (0.5 g/L). All compounds were dissolved in 1 L of distilled water and sterilized at 121 ºC for 21 min with a final pH of 6.0.

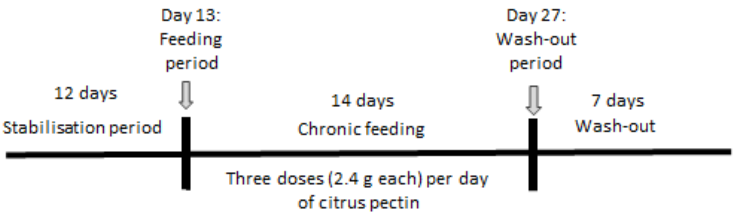

Figure 1. Schematic representation of the experimental protocol of the simgi® trial developed to assess the gastrointestinal digestion and fermentation properties of citrus pectin (30 g/L in nutritive medium)

The ascending, transverse and descending colon compartments were filled and pre-conditioned with the nutritive medium that feed the system during the stabilization period; 250 mL (AC), 400 mL (TC) and 300 mL (DC) of nutritive medium were added and later inoculated with 20 mL of fresh faecal slurry (20% w/v).

A stabilisation period of 12 days was applied to allow the intestinal microbiota to adapt to environmental conditions present in the colon compartments and to form a stable microbial community (Barroso et al. 2015). This stabilisation was approached by feeding the small intestine with nutritive medium (75 mL, pH 2) mixed with pancreatic juice (40 mL of a solution of 12 g/L NaHCO3 (VWR Chemicals), 6 g/L oxgall dehydrated fresh bile (DifcoTM, BD) and 0.9 g/L porcine pancreatin (Sigma-aldrich) three times a day during 12 days (Van Den Abbele et al. 2010). After stabilisation period of the colonic microbiota, the simgi® was subjected to a 2-week experiment, which consisted of adding 240 mL of the commercial citrus pectin per day (3 doses of 80 mL) dissolved in the feeding nutritive medium (30 g/L, pH 3.1). This sample was added directly to the stomach during 14 days, where it was mixed with gastric electrolytes and pepsin by the simulated peristaltic moves, controlling the decrease of pH by adding 0.5 M HCl. After stomach digestion, stomach content was automatically transferred to the small intestine vessel where digestion was performed during 2 h at 37 °C (pH = 7.0). Then, this content was transferred to the following compartment (AC) at a flow rate of 5 mL/min, which simultaneously activated the transit of colonic content between the AC, TC and DC compartments at the same flow rate. The temperature (37 °C), continuous flushing of nitrogen and pH were continuously controlled by the

system. pH in the colonic units was controlled by addition of 0.5 M NaOH and 0.5 M HCl to keep values of 5.6 ± 0.2 in the AC, 6.3 ± 0.2 in the TC and 6.8 ± 0.2 in the DC. Finally, a 1-week washout period was included at the end of the experiment by feeding the simgi® daily with nutritive medium. During the whole study, samples were collected every day at regular time points from the three colon vessels: During stabilisation period (< Day 13, and immediately prior to pectin feeding (Day 13*), during pectin feeding period, samples were also taken in stomach and small intestine compartments (Day 13-27) and after the beginning of washout period (Day 27- 34). Finally, all collected samples were immediately centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatants were aliquoted and stored at – 20 °C until being analysed for short-chain fatty acids (SCFA), ammonium and molecular weight estimation of the tested pectin. Microbial plate count analyses were performed at the time of the sample collection. Pellets were stored at – 80 °C until further analysis of total bacteria and main bacterial groups by *q*PCR.

Estimation of the molecular weight (Mw) of pectin. The estimation and monitoring of Mw of pectin samples during the gastrointestinal digestion with the simgi® was carried out by Size Exclusion Chromatography (SEC), according to the method described by Muñoz-Almagro et al. (2018b). Analysis was carried out on a LC Agilent Technologies 1220 Infinity LC System 1260 (Agilent Technologies, Boeblingen, Germain), equipped with

two consecutive TSK-GEL columns (G5000 PW_{XL}. 7.8 x 300 mm, particle size 10 μm, G2500 PW_{XL}, 7.8 x 300 mm, particle size 6 μm; Tosoh Bioscience, Stuttgart, Germany). Centrifuged samples from the different compartments were first diluted before HPLC analysis: 1/10, 1/4 and 1/2 in HPLC water for ST, SI and AC, TC, DC compartments, respectively. Diluted samples were filtered and eluted (20 μL) with 0.1 M $NH_4CH_3CO_2$ at a flow rate of 0.5 mL/min for 80 min at 30 °C. The eluent was monitored with an Evaporative Light Scattering Detector (ELSD) (Boeblingen, Germain) at 30 °C. Pullulans of Mw 805, 200, 10, 3 and 0.3 kDa were used as standards to calibration. All Mw values specified were weight-average.

Short-chain fatty acids (SCFA) analysis. SCFA analysis was performed by liquid chromatography using a UV-975 detector following the method described by Sanz et al. (2005)**.** Briefly, samples from the different colon compartments (AC, TC and DC) were filtered and injected on a HPLC system (Agilent Technologies, Germany) equipped with a UV-975 detector and automatic injector. SCFA were separated using a Rezex ROA Organic Acids column (300 x 7.8 mm) (Phenomenex, Macclesfield, UK) thermostated at 50 °C. Mobile phase was sulphuric acid 0.005 mM in HPLC grade water at a flow rate of 0.5 mL/min under isocratic elution. The elution profile was monitored at 210 nm and peaks were compared to standards to be identified. Data acquisition and integration were done using Agilent ChemStation

software (Wilmington, DE, USA). Calibration curves of all SCFA were obtained from the analysis of standard solutions of lactic, formic, acetic, propionic, butyric, valeric and isovaleric acid, ranging the concentrations of 1-100 mM.

Ammonium determination. Ammonium levels were determined using the Ammonium test (Spectroquant Ammonium Test, Merck), following the manufacturer's instructions. Briefly, serial dilutions of an ammonium standard solution (10 g/L) were used to prepare calibration curves. Simgi® samples were diluted with deionized water (1:10). Just prior to performing the measurement at 25 ºC, 5 mL of reactive NH4-1 and reactive NH4-2 were added to the diluted standards or samples. The mixture was shaken between each reagent addition. Then, the absorbance was quantified at 690 nm. Analyses were performed in duplicate. The results were expressed as mg of NH_4^+ contained in each colon compartment.

Microbial analyses:

Plate counts. Collected samples from the different colon compartments were diluted (1/10) in a physiological solution (0.9 %) and were plated on eight types of genera and selective media as follows: Tryptic Soy Agar (TSA) (Becton and Dickson & Company, BD) for total aerobes; Wilkins-Chalgren agar (BD) for total anaerobes; MacConkey agar (BD) for *Enterobacteriaceae;* Enterococcus agar (BD) for *Enterococcus* spp.*;* MRS

agar (Pronadisa) for lactic acid bacteria and Tryptose Sulfite Cycloserine agar (TSC) (Pronadisa) for *Clostridium* spp. Plates were incubated at 37 °C for 24-48 h in an anaerobic chamber (BACTRON Anaerobic/Environmental Chamber, SHELLAB, USA), except for TSA which was incubated in aerobic conditions (Nüve Incubator EN 120, NÜVE, Turkey).

Bacterial DNA extraction and quantitative polymerase chain reaction (*q*PCR). Bacterial DNA extraction of pellets from AC, TC and DC compartments was performed using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's recommended protocol. Extracted DNA of all samples was stored at -80 °C until analysis.

The amplification and detection of bacterial DNA was carried out on a ViiA7 Real-Time PCR System (Applied Biosystems). Specific 16s rRNA-targeting primers were used in this study to determine total bacteria, *Bacteroides* spp, *Bifidobacterium* spp, *Enterobacteriaceae, Enterococcaceae, Lactobacillus* spp, *Faecalibacterium prausnitzii* and the *Clostridium cluster XIVa.* Reactions were done in triplicate in 384-well plates using SYBR® Green PCR Master Mix (Applied Biosystems). Final volume of each amplification reaction was 10 µL: 5 µL of SYBR® Green, 0.3 µL of each primer (10 µM), 3.4 µL of nuclease-free water purified for PCR (Sigma-Aldrich) and 1 µL of DNA template. Thermal cycling consisted of an initial cycle of 95 °C, 3 min, followed by 40 cycles of 95 °C, 15 s and 1 min at the appropriate primer-pair

temperature (Gil-Sánchez et al. 2017). In order to quantify bacterial groups, DNA isolated from selected bacterial strains was used, *Bacteroides fragilis* for *Bacteroides, Bifidobacterium longum* for *Bifidobacterium, Blautia coccoides* for *Clostridia XIVa, F. prausnitzi* for *Faecalobacterium prausnitzi, Escherichia coli* for *Enterobacteriaceae, Enterococcus falcium* for *Enterococcaceae, Lactobacillus plantarum* for *Lactobacillus* and *B. fragilis* for *total bacteria*. Standard curves were generated by plotting threshold cycles (CT) vs. bacterial quantity expressed as colony-forming units (CFU)/mL.

Statistical analysis. Statistical analysis was performed using SPSS for Windows, version 23.0. One-way analysis of variance (ANOVA) and Tukey's post hoc test was used to determine significant differences among the bacterial group populations obtained after the qPCR analysis and organic acid concentrations to test the main effects of factors studied (time, pectin feeding, compartment). Differences were considered significant at $p < 0.05$ ($n = 3$).

Results:

Characterisation results showed GalA as the main component with 66.5 ± 0.2 % of the total carbohydrates; galactose was the second main component with 20.2 ± 0.1 % and rhamnose and arabinose were present with 5.8 and 3.5 %,

respectively. Glucose, mannose and xylose were also determined in minor values, 1.8, 1.4 and 0.9 %, respectively. Interval of Mw and methoxylation degree of pectin were determined as 100-800 kDa (average 350 ± 30 kDa) and 70.7 %, respectively (Ferreira-Lazarte et al. 2018).

Effect of digestion and fermentation on pectin molecular weight. The behaviour of pectin during the chronic feeding period (**Figure 1**) was evaluated by monitoring the Mw during its passage through the different compartments (ST, SI, AC, TC and DC). **Table 1** shows the quantitative results obtained by SEC-ELSD determination. Analyses were carried out just before starting the feeding period at day 13 (representing the nutritive medium), and three random days during the chronic feeding (Day 15, 24 and 27) as well as the last day of the washout period (Day 34). Results showed a high average Mw for citrus pectin (350 ± 30 kDa) which represented almost 54 % of total content when mixed with nutritive medium, whereas the latter was mainly constituted of low Mw carbohydrates (<18 kDa). Values at Day 13* (before feeding with citrus pectin) showed almost no changes between all compartments. During the chronic feeding, pectin showed no changes in the stomach compartment when compared to the intact pectin (before feeding the system), whereas a slight decrease can be observed after the small intestine passage, showing a high resistance of citrus pectin to the upper gastrointestinal digestion.

Table 1. Effect of the gastrointestinal digestion (simgi®) on the estimation and distribution of Mw (Average Mw) of the studied pectin (3%, w/v).

		Concentration of carbohydrate fraction (%)		
Sample/Day	Compartment	350 ± 30 kDa	40 ± 5 kDa	< 18 kDa
Medium	-	-	6.0 ± 1.5	94.0 ± 0.0
Medium+Pectin	-	53.6 ± 1.2	-	46.4 ± 0.0
Day 13*	ST	-	5.7 ± 0.5	94.2 ± 0.1
	SI	-	6.9 ± 0.1	93.0 ± 0.3
	AC	-	5.0 ± 0.2	94.9 ± 0.2
	TC	-	4.2 ± 0.1	95.8 ± 0.1
	DC	-	4.3 ± 0.0	95.5 ± 0.0
Day 15	ST	56.6 ± 0.6	-	42.8 ± 0.9
	SI	52.9 ± 0.4	-	46.7 ± 0.5
	AC	8.5 ± 0.6	15.1 ± 0.8	75.8 ± 0.6
	TC	9.5 ± 0.2	4.8 ± 0.1	85.2 ± 0.3
	DC	9.8 ± 0.1	4.6 ± 0.1	85.1 ± 0.1
Day 24	ST	56.5 ± 1.5	-	42.0 ± 0.3
	SI	47.6 ± 0.3	-	55.9 ± 0.7
	AC	5.4 ± 0.1	17.2 ± 0.9	76.9 ± 0.1
	TC	4.8 ± 0.4	3.5 ± 0.0	91.4 ± 0.5
	DC	4.7 ± 0.2	2.2 ± 0.0	92.8 ± 0.3
Day 27	ST	55.8 ± 0.9	-	43.5 ± 0.2
	SI	47.7 ± 0.8	-	55.7 ± 0.6
	AC	1.9 ± 0.0	17.7 ± 0.6	79.7 ± 0.1
	TC	0.6 ± 0.0	3.7 ± 0.1	95.5 ± 0.1
	DC	0.5 ± 0.0	2.5 ± 0.0	96.8 ± 0.0
Day 34	ST	9.8 ± 0.0	-	89.7 ± 0.4
	SI	7.0 ± 0.1	-	92.8 ± 0.7
	AC	4.0 ± 0.0	-	95.8 ± 0.6
	TC	3.9 ± 0.0	-	96.0 ± 0.2
	DC	2.0 ± 0.0	-	98.0 ± 0.0

Data are expressed as the mean ± SD (n=2).
*Sample taken before feeding with citrus pectin.

However, fermentation in the three different sections of colon gave rise to a remarkable effect on pectin Mw. At this stage, it can be seen the presence of

a new chromatographic peak of lower Mw (40 ± 5 kDa) than the peak corresponding to the intact pectin (350 ± 30 kDa), as well as an increase in the abundance of the peak including low Mw carbohydrates (<18 kDa), probably due to the fermentation of pectin (**Figure 2**). Lastly, washout period showed almost no presence of any carbohydrates since feeding with nutritive medium/pectin was substituted with only nutritive medium (**Table 1**).

Evolution of the microbial community. The computer-controlled multicompartmental dynamic gastrointestinal model used in this study, allowed us to monitoring the gastrointestinal digestion and fermentation in the different compartments/sections due to its capability to simulate *in vitro* the microbial conditions that characterize the different regions of the gut.

***q*PCR analysis.** Given that important modifications in the HPLC profiles of pectin were found together with slight trends observed by the plate counts (Table 2S, *Annex C*), a qPCR analysis was done at the last day of each period in the AC, TC and DC compartments in order to better assess changes in the microbial population during the *in vitro* fermentation of citrus pectin (**Table 2**). In general, higher amounts of bacteria were obtained with qPCR as compared with plate counts, which is in consonance with the fact that only a small fraction of the range of gut bacterial groups found had been, up to now, cultured (Zoetendal et al. 2006).

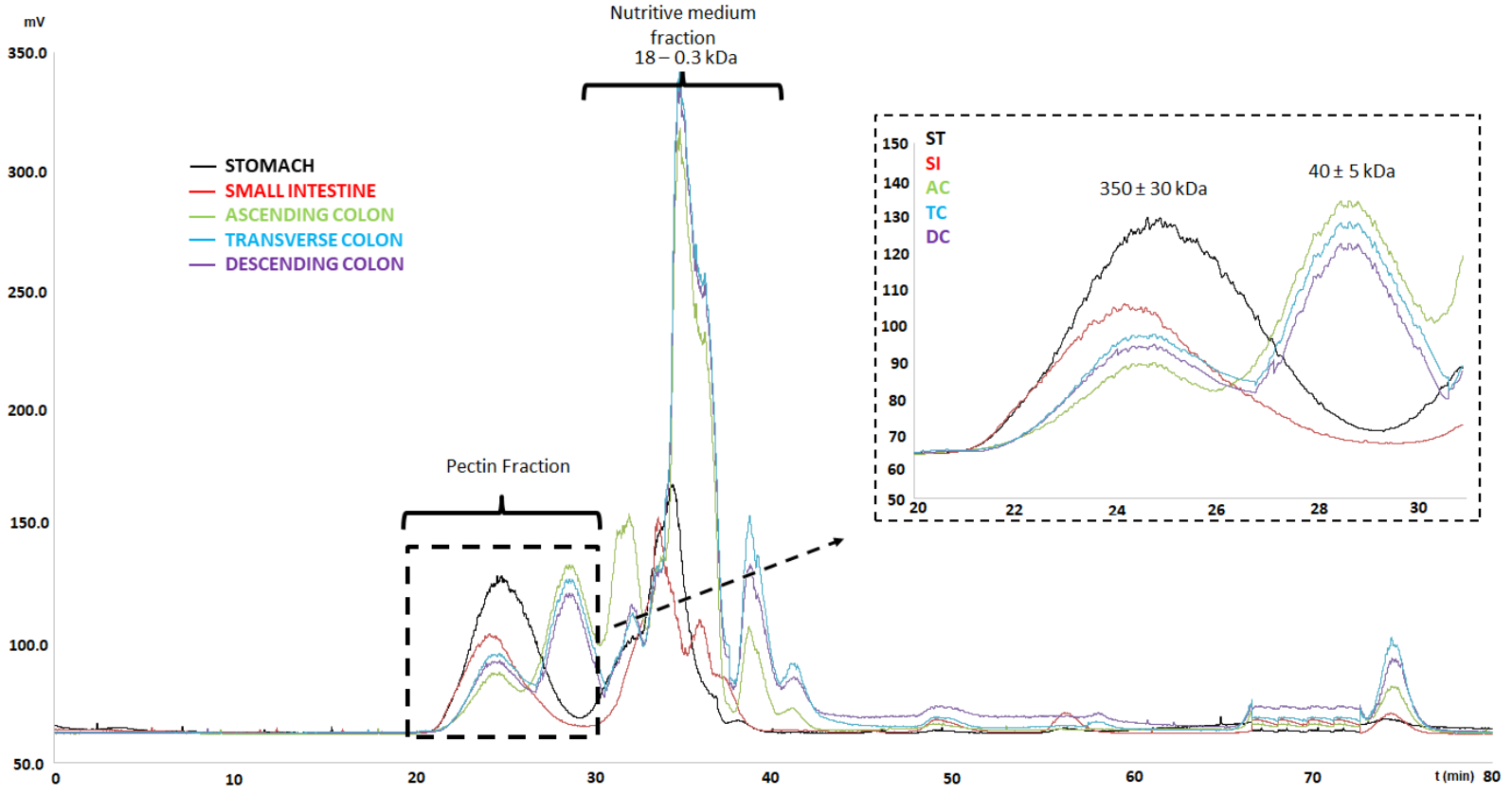

Figure 2. Qualitative evolution of citrus pectin (Mw distribution) in the five compartments (Stomach (dilution 1/10), Small Intestine (1/4), Ascending Colon (1/2), Transverse Colon (1/2) and Descending Colon (1/2)) during feeding of the dynamic simulator of the gastrointestinal tract (simgi®).

The rest of bacteria were often labelled as “unculturable” due to the generally fastidious anaerobic growth requirements (Allen-Vercoe 2013). Counts of total bacteria group were about 9.5 log copy number/mL at the end of stabilisation period and increased significantly ($p < 0.05$) after citrus pectin feeding in AC and TC whereas in DC a lower and non-significant increase ($p > 0.05$) was determined. In addition, a decrease was observed in all compartments after washout period.

Among all bacterial groups that were studied, a huge and significant increase in counts of *Bifidobacterium* spp, *Bacteroides* spp, *F. prausnitzii* and *Enterobacteriaceae* was observed after the feeding with the citrus pectin in all colon sections with the exception of *Enterobacteriaceae* in TC. Furthermore, an overall and statistically significant decrease was found after the washout period, with some exceptions (i.e., *Bacteroides* spp in AC, or *Enterobacteriaceae* in all colon sections). In contrast, a decrease in *Lactobacillus* spp (in all colon sections) and *Enterococaceae* (in TC) was also observed after the feeding period with pectin. These values increased during the washout period for *Enterococaceae* whereas did not present any changes in *Lactobacillus* spp.

Table 2. Mean values (n = 3) of the qPCR data as copy number/mL for the microbial groups analysed in the ascending (AC), transverse (TC) and descending colon (DC) of the dynamic gastrointestinal model (simgi®) at the end of stabilization period (day 13), chronic intake (day 27) and washout period (day 34) with citrus pectin. Values in brackets represents the data as the $\log_{10}$ of copy number/mL

Bacteria group	Compartment	Stabilisation period Day 13*	Chronic intake period Day 27	Washout period Day 34
Total bacteria	AC	$3.1x10^9$ (9.49 ± 0.05)^a	$6.6x10^9$ (9.82 ± 0.02)^c	$4.8x10^9$ (9.68 ± 0.04)^b
	TC	$3.4x10^9$ (9.50 ± 0.23)^a	$1.5x10^{10}$ (10.16 ± 0.01)^b	$3.0x10^9$ (9.48 ± 0.06)^a
	DC	$2.7x10^9$ (9.43 ± 0.01)^a	$3.9x10^9$ (9.59 ± 0.02)^a	$1.6x10^9$ (9.14 ± 0.33)^a
Lactobacillus	AC	$1.8x10^5$ (5.24 ± 0.13)^b	$\leq 10^4$ ($\leq$ 4)^a	$\leq 10^4$ ($\leq$ 4)^a
	TC	$6.7x10^5$ (5.79 ± 0.24)^b	$\leq 10^4$ ($\leq$ 4)^a	$\leq 10^4$ ($\leq$ 4)^a
	DC	$3.6x10^5$ (5.53 ± 0.18)^b	$2.8x10^4$ (4.39 ± 0.28)^a	$1.5x10^4$ (4.03 ± 0.48)^a
Bifidobacterium	AC	$2.8x10^5$ (5.44 ± 0.04)^a	$3.7x10^8$ (8.56 ± 0.06)^c	$2.8x10^6$ (6.43 ± 0.13)^b
	TC	$7.6x10^5$ (5.87 ± 0.14)^a	$1.1x10^8$ (8.02 ± 0.09)^c	$6.0x10^6$ (6.77 ± 0.05)^b
	DC	$3.2x10^5$ (5.50 ± 0.07)^a	$3.4x10^8$ (8.54 ± 0.02)^c	$9.6x10^6$ (6.98 ± 0.02)^b
Bacteroides	AC	$2.8x10^8$ (8.45 ± 0.08)^a	$1.7x10^9$ (9.24 ± 0.01)^b	$1.5x10^9$ (9.18 ± 0.02)^b
	TC	$7.0x10^8$ (8.85 ± 0.01)^a	$4.4x10^9$ (9.64 ± 0.03)^b	$6.2x10^8$ (8.79 ± 0.07)^a
	DC	$3.4x10^8$ (8.53 ± 0.05)^a	$8.5x10^9$ (8.93 ± 0.02)^c	$5.0x10^8$ (8.70 ± 0.02)^b
Faecalobacterium prausnitzii	AC	$\leq 10^5$ ($\leq$ 5)^a	$2.8x10^7$ (7.43 ± 0.14)^b	$\leq 10^5$ ($\leq$ 5)^a
	TC	$\leq 10^5$ ($\leq$ 5)^a	$1.3x10^8$ (8.08 ± 0.18)^b	$\leq 10^5$ ($\leq$ 5)^a
	DC	$\leq 10^5$ ($\leq$ 5)^a	$1.6x10^7$ (7.17 ± 0.21)^b	$1.1x10^5$ (5.05 ± 0.10)^a
Enterococaceae	AC	$\leq 10^4$ ($\leq$ 4)^a	$4.9x10^5$ (5.68 0.06)^b	$\leq 10^4$ ($\leq$ 4)^a
	TC	$6.8x10^6$ (6.83 ± 0.05)^b	$1.8x10^4$ (4.19 0.28)^a	$2.5x10^6$ (6.40 0.02)^b
	DC	$1.1x10^6$ (6.05 ± 0.04)^a	$3.2x10^5$ (5.50 0.03)^a	$6.4x10^5$ (5.69 0.45)^a
Enterobacteriaceae	AC	$9.5x10^6$ (6.98 ± 0.02)^a	$4.6x10^8$ (8.66 ± 0.03)^b	$3.6x10^8$ (8.55 ± 0.07)^b
	TC	$2.1x10^8$ (8.32 ± 0.08)^{ab}	$1.7x10^8$ (8.21 ± 0.19)^a	$4.1x10^8$ (8.60 ± 0.07)^b
	DC	$5.6x10^7$ (7.70 ± 0.24)^a	$1.9x10^8$ (8.23 ± 0.25)^b	$2.3x10^8$ (8.37 ± 0.02)^b

^{a,b,c} Significant differences ($p < 0.05$, ANOVA) were determined for $\log_{10}$ values (in brackets) for the same bacterial group. Letters represent significant differences between days for the same compartment in each bacterial group.
*Sample taken before feeding with citrus pectin.
Standard deviation values are in brackets.

Metabolic activity. The metabolic activity of the microbiota before, during and after feeding with pectin in the different colonic reactors of the simgi® was evaluated by monitoring the content of SCFA (fermentative metabolism) and of ammonium (proteolytic metabolism).

Ammonium determination. Evolution of ammonia during the simulation of the gastrointestinal digestion in the three colon compartments is shown in **Figure 3**.

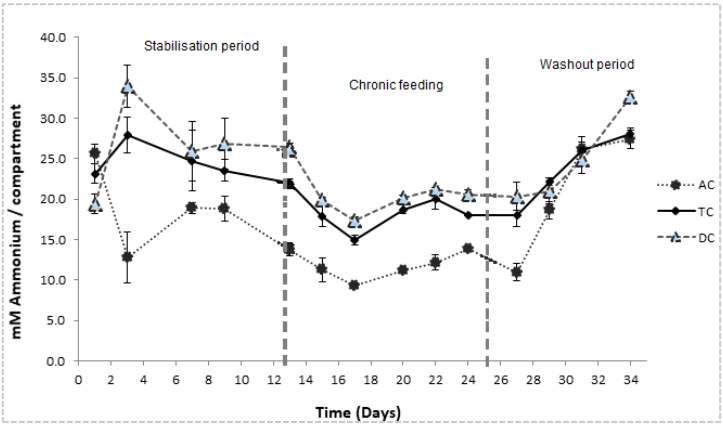

Figure 3. Ammonium Evolution (mM) during gastrointestinal digestion of citrus pectin in the simgi®

A slight but significant decrease ($p < 0.05$) in ammonium concentration is observed during the feeding with the citrus pectin, as compared with stabilisation and washout periods. Also, as it has been observed in previous studies with the same system (simgi®) (Barroso et al. 2016, Barroso et al. 2015; Gil-Sánchez et al. 2017), ammonium concentration gradually increased

from AC to the DC compartment because of the accumulation of products in the system, which lacks of any absorption steps between the different compartments. These values showed that proteolytic metabolism occurred through the entire colon compartments during the whole experiment, but it was substantially diminished during the chronic feeding period with citrus pectin.

Short-chain fatty acid (SCFA) analysis. The major end-products of indigestible carbohydrates metabolism by the colonic microbiota are SCFA. SCFA evolution (mM) during the stabilisation period, chronic feeding of citrus pectin and washout period is shown in **Figure 4.** SCFA concentrations presented no changes during the stabilisation period in all three compartments. During the chronic feeding with pectin these levels showed a significant increase in all major SCFA (acetate, propionate and butyrate) which decreased after elimination of nutritive medium/pectin administration. As expected, SCFA production consisted mainly of acetate, butyrate and propionate with small amounts of lactate and valerate in all compartments. Acetate was the most abundant SCFA, followed by butyric and propionic acid, showing increases up to 297, 92 and 60 %, respectively, after chronic feeding, as compared to the initial levels (Day 13). Afterwards, they showed a considerable decrease during washout period started.

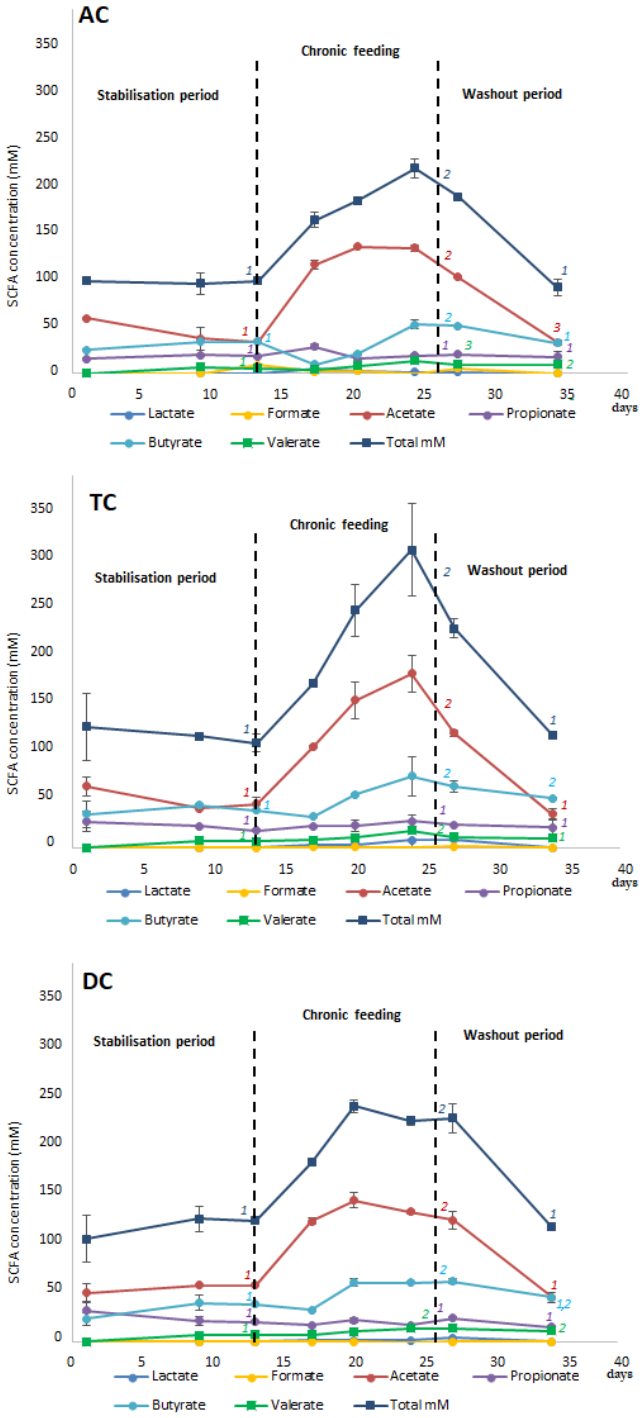

Figure 4. Evolution in concentration of SCFA in the ascending (AC), transverse (TC) and descending colon (DC) of the simgi® during the stabilisation (D1-D13), feeding (D13-D27) and washout period (D27-34) with citrus pectin solution (3 g/L). [1,2,3] Numbers represent differences ($p < 0.05$) between the data at the end of each period.

Overall, the total SCFA average molar production was compartment-dependent, being higher in the transverse and descending colon. Regarding minor SCFA, results also showed significant values of valerate in all three compartments (Khodaei et al. 2016). Formic acid was detected in the AC compartment reaching values of 2.7-8.0 mM whereas TC and DC presented concentrations below 1.1 mM. Lactic acid production was only detected at fermentation days with the citrus pectin (1.3, 8.7 and 4.0 mM in ascending, transverse and descending colon, respectively). Although lactate is not a SCFA, it is usually considered in the metabolism of bacteria as a product of saccharolytic fermentation. Furthermore, valerate has been described as a primary end product of lactate fermentation (Almeida et al. 2017; Unger et al. 2016; Yoshikawa et al 2018).

Discussion:

Several studies have shown that oligosaccharides deriving from pectins exert bifidogenic activities. Furthermore, there are also studies that have demonstrated a significant growth of bacteria in intact pectins suggesting a potential role of this polysaccharide as a prebiotic (Ferreira-Lazarte et al. 2018; Gómez et al. 2016; Yang et al. 2013). In fact, in a static *in vitro* study, we have recently shown that either pectin or enzymatically-modified pectin from different by-products stimulates beneficial bacteria of colonic

microbiota (Ferreira-Lazarte et al. 2018). However, no investigation has been carried out on the properties of pectin as a substrate for fermentation including a previous passage through the upper gastrointestinal tract. This study remarks, for the first time, the use of the simgi® to evaluate the effect of the upper gastrointestinal digestion on a commercial citrus pectin and its effect on colonic microbiota metabolism.

Pectin taken from the stomach compartment showed almost no changes when compared to the initial status, whereas samples taken from the small intestine revealed some loss of pectin, 6.5, 15.7 and 14.7 % for Day 15, Day 24 and Day 27, respectively. Holloway et al. (1983) observed a loss of pectin of 15-40 % in an *in vivo* study with ileostomy samples. In the same way, Saito et al. (2005) found that approximately 90 % of ingested pectin was recovered in the terminal ileum in an *in vivo* study collecting endoscopy retrograde samples. These studies attributed the loss of pectin to the possible degradation by bacteria within the digestive tract, especially the terminal ileum. However, given that in our prototype of digestion the presence of bacteria is confined to the colon compartments, changes observed in the Mw of pectin after its passage through the SI could be related to other chemical effects due to the interaction with pancreatic fluids and bile salts (Miller et al. 1995).

Regarding the effect of digested pectin on microbiota, results obtained showed that citrus pectin favourably impacts on microbiota composition and functionality in the three compartments (AC, TC and DC) of the simgi® model.

Pectin fermentation produced an increase in the counts of total bacteria, compared to the initial state, with significant increases in the proximal regions (ascending and transverse colon) probably due to the content of polysaccharide coming from the small intestine. Furthermore, high methoxyl pectins, as it is the case of citrus pectin here assayed, have showed a slower fermentation in the large intestine of rats, which allows the fermentation to take part in all three compartments (Dongowski et al. 2002).

According to some authors, increments up to 0.5 - 1.0 $\log_{10}$ in *Bifidobacterium* populations could be considered as a major shift in the gut microbiota towards a potentially healthier composition of intestinal microbiota (Kolida & Gibson 2007). *Bifidobacterium* and lactobacilli have been traditionally considered as the major microbial targets for prebiotic action, due to their beneficial effects (Roberfroid et al. 2010). Similar values of *Lactobacillus* spp and *Bifidobacterium* spp populations at the end of stabilisation period were observed (**Table 2**). Increases up to 2.15 - 3.12 $\log_{10}$ in *Bifidobacterium* group was determined in all compartments, being the highest increase of all bacteria determined. This could be attributed to the

high galactose/arabinose content of the studied pectin (23.8 %) (Di et al 2017; Onumpai et al. 2011). However, unlike *Bifidobacterium*, *Lactobacillus* group showed a significant decrease after feeding with citrus pectin. In related studies, Olano-Martin et al. (2002) reported that both POS and citrus pectin significantly increased the number of *Bifidobacteria*, whereas lactobacilli numbers only increased with POS although this increase was not statistically significant. Furthermore, Chen et al. (2013), showed an increase of *Bifidobacteria* during the *in vitro* fermentation of apple pectin (DM 70%) and POS, whereas *Lactobacillus* population presented no changes or even similar values for pectin compared to the negative control after 24 h of fermentation. Li et al. (2018) also showed a decrease of *Lactobacillus* when feeding rats with pectin extracted from citrus peels in an *in vivo* study.

Faecalobacterium prausnitzii values reported to be the second highest increase during the fermentation of pectin in the simulator (2.17 – 3.03 $\log_{10}$). *F. prausnitzii* is one of the most abundant commensal bacteria in the healthy large intestine and is one of the main producers of butyrate in the human colon (Louis et al. 2007; Louis et al. 2014). Furthermore, low *F. prausnitzii* levels were correlated with the recurrence of inflammatory bowel disease and it has confirmed to have anti-inflammatory effects (Onumpai et al. 2011; Sokol et al. 2008). Likewise, it has been suggested that this bacterium could be a good probiotic candidate to counterbalance dysbiosis in Crohn's disease

patients (Scott et al. 2014; Sokol et al. 2009). Moreover, previous studies have shown that this bacterium could have a major role in pectin utilization in comparison with other two abundant pectin-utilizing species, *Bacteroides thetaiotaomicron* and *Eubacterium eligens* (Lopez-Siles et al. 2012).

Bacteroides population showed also a high increase being the third highest increase of all bacteria determined with values of 0.4 – 0.8 $\log_{10}$. *Bacteroides* are one of the enterotypes of the human microbiota, which are responsible for the major part of polysaccharide digestion occurring in the human large intestine (Flint et al. 2012; Salazar et al. 2009). In fact, many strains from human faeces can produce various pectinolytic enzymes, including polygalacturonase, pectin methylesterase, extracellular and cell-associated pectate lyase (Dekker & Palmer, 1981; Jensen & Canale-Parola 1986). Hence, *Bacteroides* could be involved in cross-feeding with *Bifidobacteria* by releasing breakdown products of pectin which might be utilized by the latter.

Enterococaceae and *Enterobacteriaceae* groups presented different behaviour compared to the bacteria mentioned before. Significant increase were found in the AC for both bacteria during the feeding period with citrus pectin, whilst TC showed a decrease of *Enterococaceae* and no significant change for *Enterobacteriaceae*. Nevertheless, a significant increase in

Enterobacteriaceae population was found in DC whereas stable levels were observed for *Enterococaceae* after feeding with citrus pectin.

Concerning the proteolytic and saccharolytic activity of microorganisms, SCFA concentrations increased during the chronic feeding with pectin. Lactic and formic acid were observed in low concentrations since produced lactic acid is considered to be an intermediate metabolite and can be further metabolized within the colon and turned into butyric and propionic acids through cross-feeding by gut bacteria (Duncan et al. 2004; Reichardt et al. 2014). Similarly, formic acid is used by microorganisms, which have a particularly important role in anaerobic metabolism, via interspecies cross-feeding interactions (Louis et al. 2014). Results obtained showed an increase for valerate during the pectin fermentation. Khodaei et al. (2016) also reported a small amount of valerate when testing a galactose/rhamnose rich polysaccharide with similar values compared with recognised prebiotic, such as FOS.

The major end-products of saccharolytic fermentation are acetate, propionate and butyrate, which have a combined concentration of 50-150 mM in the colon (Louis et al. 2014). High levels of SCFA are desirable since, among other benefits, the corresponding decrease in the pH values can suppress the growing of pathogenic bacteria. **Figure 4** shows a significant high increase of

these compounds, being acetate the major SCFA produced followed by butyric and propionic, respectively.

Given the complexity of the human microbiota, it is challenging to attribute a particular fermentation end-product to a specific bacterial group, however, acetate is typically generated via *bifidus* pathway, and more specifically it is a major end-product of *Bifidobacterium* fermentation (Sanz et al. 2005). Thus, the high production of acetate observed in our study can be ascribed to the growth of *Bifidobacterium* population in presence of pectin. The high increase of propionate concentrations after feeding the system with pectin is in good agreement with the increase in *Bacteroides* population, one of the main propionate-producing bacteria in the human colon. Propionate has also been shown to exert beneficial effects such as protective role against carcinogenesis through the decrease in human colon cancer cell growth (Hinnebusch et al. 2002; Jan et al. 2002). In addition, propionate and formate were reported to reduce the activity of *E. coli* and *Salmonella* at pH 5 (Gullón et al. 2011; Topping & Clifton 2001). Significant increases in butyrate concentrations were also observed in all three compartments, with the second highest levels after acetate. *F. prausnitzii* might utilise apple, citrus and sugar beet pectin as a source of growth and butyrate formation as shown by using pure cultures and *in vitro* models (Lopez-Siles et al. 2012; Chung et al. 2017; Onumpai et al 2011; Gómez et al. 2016). Thus, butyrate levels concur with

the high increase of *F. prausnitzii* population observed. Furthermore, higher levels of butyrate can also be explained due to cross-feeding between *Bifidobacteria* and *F. prausnitzii* since the latter is able to use the acetate produced by *B. adolescentis* thereby boosting butyrate formation (Rios-Covian et al. 2015). Apart from these effects, butyrate is known to affect several components of the colonic defence barrier, resulting in enhanced protection against luminal antigens (Hamer et al. 2008; Havenaar 2011).

Regarding ammonia concentration, **Figure 3** showed a slight but significant decrease in ammonium concentration during the feeding with citrus pectin. It is noteworthy that lower proteolytic activities are usually associated with health-promoting effects (Ichikawa & Sakata 1998), since it can be a potential carcinogenic agent at relatively low concentrations, as has been shown by the increase in mucosal damage and colonic adenocarcinoma in a rat model (Louis et al. 2014; Windey et al. 2012). A significant positive correlation was observed between SCFA levels and ammonia excretion where more acidic conditions favour the excretion of ammonia due to the protonation and formation of poorly absorbed ammonium ion (Louis et al. 2014).

Conclusions:

The *in vitro* study of citrus pectin using the dynamic gastrointestinal simulator simgi® pointed out its high indigestibility, since a reduced hydrolysis of pectin (~12%) was detected in the upper gastrointestinal tract (ST and SI), mainly due to chemical interactions with pancreatic fluids and bile salts. Findings also highlight the important role played by pectin in stimulating beneficial bacteria such as *Bifidobacteria, F. prausnitzii* and *Bacteroides* (especially in the first two bacterial groups). A high increase in acetate, propionate and butyrate concentration was observed due to fermentation of pectin by the microbiota but also to cross-feeding interactions between different bacteria. Increase in SCFA also produced a decrease in ammonia concentration, which is associated with health-promoting effects. This is the first study of gastrointestinal digestion and fermentation of pectin in a dynamic gastrointestinal simulator and, although further *in vivo* studies should be conducted, the data obtained confirmed the potential of pectin to be considered shortly as emergent prebiotics with a possible use for human consumption.

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GENERAL DISCUSSION

5. General Discussion

As was already underlined in the previous sections, the scientific interest towards the regulation of the gut microbiota has considerably grown due to its relationship with health and several important diseases. In this regard, the ability of prebiotic ingredients to modulate the composition and/or activity of the gut microbiota has received the greatest attention. However, given that prebiotic properties such as their resistance to digestion or the ability to be fermented by specific bacteria, will depend on their structure (degree of polymerization, linkage types, monosaccharide composition, etc.), it is important to have solid knowledge about/on possible changes that these compounds can suffer before reaching the colon. Moreover, establishing the specific structure characteristics of oligosaccharides that have a direct impact on their resistance to digestion, could allow us to extend the knowledge for the sake of a future potential development of new tailored prebiotics. On the other hand, the interest towards amplifying the number of prebiotic compounds had led to the search of new and/or improved methods and sources of obtainment. In this sense, agro-food waste by-products could represent a more sustainable and promising source for prebiotics obtainment.

In this sense, given the limitations of the current standardised digestion protocols for carbohydrates digestion, the first approach of this

Thesis was the search and the set up of a specific, easy and reproducible method for carbohydrate digestion using extracts derived from mammals such as that of small intestine of rats (RSIE) (*Chapter 1*). Simmilarity between intestinal rat disaccharidases activity (maltase, sucrase, palatinase. trehalase and lactase) and degradation of dietary digestible carbohydrates after intestinal digestion in rats and humans endorse the suitability of these mammalian extracts (Oku et al. 2011). Therefore, the digestion rate of recognised and potential prebiotics was assessed with the proposed method. As expected, all prebiotic and potential prebiotic compounds (GOS, FOS, lactulose, OsLu and lactosucrose) 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 on resistance to degradation. $\beta(1\rightarrow6)$ linkages in carbohydrate mixtures showed a significantly higher resistance when compared to $\beta(1\rightarrow4)$ linkages, which were the most susceptible to degradation in all samples of prebiotics 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 (β-Gal-(1→4)-Fru) and FOS (β-Fru-(2→1)-Fru_n-Glu) exhibited the highest resistance compared to total oligosaccharides (the sum of di-, tri-, and tetrasaccharides) from GOS

and OsLu, due to their monomer composition and high resistant linkages. However, molecular weight in OsLu, which has been related with a slower fermentation by the colonic microbiota than lactulose being able to reach the distal colon without great alterations (Cardelle-Cobas et al. 2011; Cardelle-Cobas et al. 2012), seems to also provide a higher resistance to intestinal enzymes. In this sense, trisaccharide fraction (mainly *β*-Gal-(1→6)-*β*-Gal-(1→4)-Fru) present in OsLu was almost no degraded (1.4 %) compared with disaccharides present in the mixture (32 %) and lactulose (~11.5 %) after *in vitro* digestion. In contrast, trisaccharide fraction in GOS mainly *β*-Gal-(1→4)-*β*-Gal-(1→4)-Glu and *β*-Gal-(1→6)-*β*-Gal-(1→4)-Glu did not showed this behaviour due to different monomeric composition in the structure, 32.7 and 11.9 % degradation, respectively.

Thus, although relationship between structure of GOS and resistance to mammalian intestinal enzymes and their bioactive effect was pointed out in previous reports (Hernández-Hernandez et al. (2012); Rastall et al. (2005)), no comparison of the digestibility of different types of GOS from lactose and lactulose using an intestinal extract from rats was carried out before.

Moreover, given that the mixtures of prebiotics are usually included in different foodstuffs, mainly milk and dairy products, the need to study the effect of the food matrix on prebiotics (lactulose, GOS and OsLu)

digestibility was considered. Therefore, the *in vitro* method indicated above was used after a previous gastrointestinal digestion following a standardised semidynamic method under simulated physiological conditions (Minekus et al. 2014) (*Chapter 2*). Firstly, electrophoretic analysis demonstrated that the presence of the studied prebiotics (at required prebiotic doses) does not affect the digestion of proteins. Moreover, the use of pancreatic fluids and bile salts (Infogest protocol) to simulate intestinal digestion evidenced the limitation of these models for carbohydrate digestion due that limited modifications were observed in the carbohydrates fraction of the samples. However, the *in vitro* proposed method using the RSIE to digest samples after their gastric stage demonstrated its suitability to hydrolyse carbohydrates showing considerable decreases in this fraction. Firstly, higher amounts of lactose did not seem to affect the prebiotics degradation after 2 hours of digestion with the RSIE. Degradation found in the oligosaccharides fraction of GOS (35 %) and OsLu (15 %) within the milk was similar to the obtained in *Chapter 1*, 34 and 18 %, respectively. In contrast, higher degradations of lactose were found due to the highest initial content in milk samples. Moreover, addition of prebiotic to milk showed to increase the degradation of free lactose in these samples with 75-83 % lactose degradation compared to milk samples without prebiotics added (61 % lactose degradation) which could contribute to a better degradation of lactose in this products when limited lactase activity is present

(Corgneau et al. 2017). Nevertheless, high decreases of lactose content were observed whereas lactulose remained as the less prone to degradation disaccharide. In line with the results of *Chapter 1*, oligosaccharides from predominant $\beta(1\rightarrow4)$ GOS mixture exhibited the highest degradation after intestinal digestion, whereas oligosaccharides from $\beta(1\rightarrow6)$ OsLu stood as the most resistant structure with 85 % of composition intact after 2 hours 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 intestine extract is needed to cover all the potential structures of carbohydrates that can reach the gastrointestinal system. Rat intestinal enzymes provided a useful and reliable tool to determine digestibility of dietary carbohydrates. However, despite the positive results obtained, small size of these animals creates a challenge for their use as human disease models due to the very different anatomy and physiology compare to humans. Thus, pigs have emerged as an important model due to their anatomical, physiological similarity to human and human genome (98 %) (Humphray et al. 2007), as well as their broad availability, short generation interval, larger litter size, and the fact that they are a food source that avoids ethical concerns (Kuzmuk & Schook 2011). The use of pigs has proved to be a robust model for several studies such as the 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 & Schook, 2009; Jensen et al. 2010; Schook et al. 2015). Moreover, 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 et al. 2013). Therefore, the use of small intestine extracts from pigs could represent a more reliable method as well as a step forward towards the development of a robust and comparable to human method to gathering information about dietary carbohydrate digestion.

Intestinal disaccharidases are identified as glycoproteins that maintain a structural linkage with elements of the apical membrane and are actively budded off as brush border membrane vesicles (BBMV) into the adjacent periapical space, consequently, brush border enzymes may transit to all parts of the lumen in this form of vesicles (McConnell et al. 2009). However, although proportions of BBMV that remain in the periapical space and diffuse into the lumen is not currently known, it is likely that the mucus layer overlying the ephitelia retards the egress of BBMV from the periapical space into the lumen so that a significant amount of BBMV would remain in close proximity to the intestinal mucosa (Hooton et al 2015). Therefore, BBMV obtained and purified from pig were used for the determination of the

intestinal digestibility of structurally different prebiotic oligosaccharides (GOS and OsLu) (*Chapter 3*). BBMV showed a higher enzymatic activity when compared to the commercial intestinal extract from rat mainly due to the exhaustive fractionation and purification carried out in the obtainment of the membrane vesicles. Digestibility assays reaffirmed the data obtained in previous chapters with a high resistance of $\beta(1\rightarrow6)$ compared to $\beta(1\rightarrow4)$ linkages. Moreover, predominantly $\beta(1\rightarrow3)$ linkages GOS that were also analysed, revealed the high susceptibility of these structures to intestinal disaccharidases with a 44 % degradation whereas $\beta(1\rightarrow4)$ and $\beta(1\rightarrow6)$ showed a 23 and 12 % hydrolysis, respectively, after 2 hours of digestion. A very recent study in the obtainment of GOS using BBMV have revealed the high preferably synthesis of GOS linked by $\beta(1\rightarrow3)$ compared to those linked by $\beta(1\rightarrow4)$ or $\beta(1\rightarrow6)$ (Julio-González et al. 2019). Therefore, considering that enzymes can catalyse reversible reactions in either direction (Abdul-Manas et al. 2018), data obtained at this stage support the hyphothesis that most glycosidic linkages formed when intestinal β-galactosidase act as transgalactosidase, are preferentially broken under hydrolytic conditions. In addition, specific monosaccharides composition provided a better resistance on several components of the samples such as galactosyl-galactoses, galactosyl-fructoses and specially on the trisaccharide fraction of OsLu

samples (β(1→6), 9.8 % hydrolysis after 2 hours), establishing the key role of these monomers.

All these findings reveal important structure-function relationships highlighting the strong resistance of β(1→6) oligosaccharides, especially of those derived from lactulose which have shown to be the most resistant to mammalian intestinal enzymes compared to different commercial GOS mixtures. Thus, when aiming for a potential development of new customized and new generation prebiotics, oligosaccharides derived from lactulose would represent an ideal candidate that have to be taken into account. In this sense, several evidences have also reported the technological and biological properties of these substrates (Villamiel et al. 2014; López-Sanz et al. 2015; Barroso et al. 2016; López-Sanz et al. 2018; Fernández et al. 2018) supporting their suitability to be considered as a good prebiotic candidate. Lastly, prebiotics definition maintains that these substrates should be "non-digested" by intestinal enzymes reaching the colon at least almost intact, however, findings obtained in this Thesis have revealed a considerable high degradation of some of these substrates.

Regarding the second part of this Thesis, the obtainment of new prebiotics from agro-food by-products was presented as an interest and renewable alternative to convetional sources (*Chapter 4*). After successful

extraction, obtained artichoke, sunflower and citrus pectin subjected to enzymatic treatment with a cellulase from *Aspergillus niger* produced modified pectins with different structural features (lower Mw, lower methoxylation degree, and changes in monomer composition). *In vitro* batch fermentations with human volunteers, which intends to simulate the complex diversity in the colon, ratified the potential prebiotic properties of these substrates, which were related to their structural features. The six pectic samples exerted a prebiotic effect when compared with recognised prebiotics such as FOS and lactulose. As products of metabolism, high increases of acetate followed by propionate and butyrate were observed in all samples after pectin fermentation by microorganisms. As indicated in previous sections, high levels of SCFA are desirable due to the several benefits that have been related with their presence such as, obesity regulation, suppression of colonic inflammation and carcinogenesis, glucose homeostasis, enhancement of the ileal motility, as well as a suppression of the growth of pathogenic bacteria due to the decrease of pH (Hong et al. 2005; Murugesan et al. 2018; Li et al 2017; Sivaprakasam et al 2016; Louis et al. 2014). Lower Mw seemed to provide a significant higher growth of *Bifidobacteria* and *Lactobacillus* in citrus and artichoke modified pectin compared to their corresponding intact pectin, whereas higher amounts of potential galactans chains branched to RG-I (Gal:Rha) provided a better growth of *Bacteroides*

in modified pectins from sunflower and artichoke compared to their unmodified pectins. Remarkably, a higher content of arabinose and galactose in the structure was related to a better growth in bifidobacteria in previous reports (Di et al. 2017; Onumpai et al. 2011). Therefore in line with those reports, artichoke pectin, which had the higher content of these two monosaccharides combined, was the substrate that promoted the significantly highest growth in *Bifidobacteria*, *Lactobacillus* and *Bacteroides*, similar to the obtained after FOS and inulin fermentation. In general, all studied substrates evidenced a good capability to promote growth of beneficial bacteria and to produce SCFA highlighting the suitability of agro-food by-products as a renewable source of bioactive pectin.

In this regard, pectins are generally accepted to be scarcely hydrolyzed by the gastrointestinal enzymes, however, it has been demonstrated that pancreatic enzymes as well as acidic gastric conditions might exert some hydrolysis towards methyl esters and O-acetyl esters groups in pectins (Miller et. al. 1995). Degradation of the Mw of pectin and cleavage of mono- and oligosaccharide has been also observed after successive acid and enzymatic *in vitro* hydrolysis of different pectin substrates (Mikhaleva et al. 2011). Moreover, similar to the results obtained in previous chapters concerning the important relationship between chemical

structure and function, few studies have evidenced an influence of certain structural characteristics such as methylation degree or the arabinoxylan structure on pectin fermentability (Dongowski et al. 2002; Rumpagaporn et al. 2015). Thus, given that the possible chemical and physicochemical structural changes could affect the fermentation properties of pectins, a continuous gastrointestinal digestion and fermentation of citrus pectin was carried out to evaluate pectin fermentation after its potential degradation during the upper gastrointestinal digestion (*Chapter 5).* Therefore, a dynamic gastrointestinal model (SIMGI®), which provides a better mimic of the physical processing and physiological events occurring during the digestion, was used to evaluate pectin gastric and small intestinal digestion and colonic fermentation. *In vitro* digestion of citrus pectin results emphasized the suitability of this substrate as a potential prebiotic candidate. Unmodified commercial pectin subjected to a continuous simulated gastric and small intestinal digestion, showed a slight decrease (~12%) on initial concentration before reaching colonic vessels, probably due to chemical effects produced by pancreatic fluids and bile salts (Miller et al. 1995). Moreover, some authors have reported degradation of these substrates after intestinal digestion in human subjects (Chinda et al. 2003; Saito et al. 2005) attributing degradation to the scarce microflora present in the small intestine, specially in the terminal ileum where bacterial densities can reach similar levels to

those found in the large intestine (Donaldson et al. 2016). However, SIMGI® *in vitro* model used lacks of microflora present at the small intestine, thus, changes observed can be attributed specifically to pancreatic fluids and bile salts. Fermentative and proteolytic metabolism reflected also the potential benefits of citrus pectin given the high values found of SCFA (acetate, propionate and butyrate) and diminution of ammonia levels during the feeding with citrus pectin. Furthermore, *q*PCR analysis during pectin fermentation pointed out huge and significant increases on the populations of beneficial bacteria such as *Bifidobacteria*, *Bacteroides* and the emerging probiotic candidate, *Faecalibacterium prausnitzii* which has been related with several health benefits (Martín et al 2017; Miquel et al. 2013), whereas a decrease was observed before and after citrus pectin inclusion on the system. Therefore, changes observed after small intestine digestion did not seem to affect the fermentability of the studied citrus pectin. Similar behaviour was observed during fermentation with*)* and without previous digestion passage, *Chapter 5* and *Chapter 4*, respectively where a high increase of *Bifidobacteria* and *Bacteroides* was observed. Findings obtained here upholds the potential of pectin to be considered as an emergent prebiotic as well as the suitability of agricultural byproducts to obtain functional ingredientes. Moreover, as well as GOS and OsLu, structural features play an important role on their prebiotic potential, therefore, structural diversity (Mw,

degree of methoxyl esterification) in pectin prebiotics could be possible whether arabino- and galactooligosaccharides are present in the structure.

General Conclusions

Conclusions Generales

6. General Conclusions

Results obtained in the present PhD Thesis concerning the *in vitro* digestibility and fermentability of selected prebiotics and functional carbohydrates with prebiotic potential have led to the following conclusions:

- The *in vitro* digestion model established on the utilization of rat small intestinal extract has proved to be a useful, reliable and an efficient approach to evaluate the digestibility of dietary carbohydrates (digestible and non-digestible), overcoming the limitations of the current standardised methods for *in vitro* gastrointestinal digestion.
- The combination of Infogest protocol with rat small intestinal extract was useful to assess the digestibility of galactooligosaccharides, lactulose and oligosaccharides derived from lactulose added to milk, as well as to demonstrate their resistance to gastric and pancreatic fluids and bile salts.
- Using a digestion model based on small intestinal brush border membrane vesicles (BBMV) from pig pointed out the influence of glycosidic linkages, degree of polymerization and monomer composition on the resistance to intestinal digestion of selected prebiotics.

- Galactooligosaccharides containing $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ linkages were more prone to degradation after intestinal digestion than $\beta(1\rightarrow6)$ using pig BBMV.
- Oligosaccharides derived from lactulose (OsLu) having $\beta(1\rightarrow6)$ as the predominant glycosidic linkage were more resistant to intestinal digestion than any type of galactooligosaccharides and lactulose, due to the combined effect of monosaccharide composition and linkage type.
- Pectin and modified pectins obtained from agricultural by-products of artichoke and sunflower presented a similar prebiotic potential as compared to recognized prebiotic such as inulin and FOS, highlighting the suitability of these substrates as renewable sources of bioactive compounds.
- Considering the structural characteristics of pectin (molecular weight, degree of methoxyl esterification, and monomeric composition) the most influencing factor on the bifidogenic properties was the presence of arabinose, being artichoke pectin the one that had the highest proportion of this monosaccharide.

- Citrus pectin was highly resistant to gastrointestinal digestion in a dynamic gastrointestinal simulator, and presented a high fermentability by colon microbiota producing great increases in the population of *Bifidobacteria, Bacteroides* and *Faecalibacterium prausnitzii*, and SCFA levels.

Therefore, based on all these conclusions, this PhD Thesis contributes to gain deeper knowledge on the digestibility of prebiotics. Moreover, high degradation of some subtrates studied in this Thesis challenge the belief that they reach the colon fully intact, and, although more studies are required, could suggest a possible revision of the current prebiotic concept.

6. Conclusiones Generales

Los resultados obtenidos en esta tesis doctoral enfocada hacia la evaluación de la digestibilidad y fermentabilidad de compuestos prebióticos y carbohidratos con potencial prebiótico, ha permitido alcanzar las siguientes conclusiones:

- El modelo de digestión *in vitro* basado en la utilización de un extracto de intestino delgado de rata ha demostrado ser una alternativa útil, fiable y eficiente para la evaluación de la digestibilidad de carbohidratos dietéticos (digeribles y no digeribles), subsanando las limitaciones de los métodos estandarizados actuales para la digestión gastrointestinal *in vitro*.
- La combinación del modelo de digestión Infogest con el extracto de intestino delgado de rata fue de gran utilidad para la determinación de la digestibilidad de galactooligosacáridos derivados de lactosa y lactulosa incluidos en leche, además de demostrar la resistencia de estos compuestos a los fluidos gástricos, pancreáticos y las sales biliares.
- El uso de vesículas procedentes del borde en cepillo del intestino delgado (BBMV) de cerdos demostró la influencia del tipo de enlace glucosídico, grado de polimerización y la composición monomérica

de distintos prebióticos sobre la resistencia de los mismos a la digestión intestinal.

- Los galactooligosacáridos con enlace predominante $\beta(1\rightarrow3)$ y $\beta(1\rightarrow4)$ fueron más susceptibles a la degradación intestinal, comparados con los enlaces $\beta(1\rightarrow6)$, utilizando las vesículas del intestino delgado de cerdo.
- Los oligosacáridos derivados de lactulosa (OsLu, $\beta(1\rightarrow6)$) fueron más resistentes a la degradación intestinal comparados con la lactulosa y otros galactooligosacáridos estudiados, debido a un efecto combinado de la composición monomérica y tipo de enlace.
- Las pectinas y pectinas modificadas obtenidas a partir de subproductos de alcachofa y girasol mostraron un potencial prebiótico similar a prebióticos reconocidos como los FOS e inulina, reforzando la idoneidad de estos sustratos como fuente alternativa de compuestos bioactivos.
- De acuerdo con las características estructurales de la pectina (tamaño molecular, grado de metoxil esterificación y composición monomérica), el contenido en arabinosa demostró ser el más influyente sobre las propiedades bifidogénicas, siendo predominante en la pectina procedente de alcachofa.

- La pectina de cítricos presentó una elevada resistencia a la digestión gastrointestinal llevada a cabo en un simulador dinámico y continuo, y presentó una elevada fermentabilidad en las fases colónicas produciendo grandes incrementos en las poblaciones de *Bifidobacteria*, *Bacteroides* y *Faecalibacterium prausnitzii* y en los niveles de SCFA.

Teniendo en cuenta todas estas conclusiones, el trabajo desarrollado en esta tesis contribuye a ampliar el conocimiento con respecto a la digestibilidad de los prebióticos. Además, la degradación de sustratos prebióticos observada tras su digestión intestinal, lleva a cuestionar la creencia general de que estos compuestos son capaces de llegar intactos al colon, sugiriendo, por lo tanto, una posible revisión de la definición de prebióticos.

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ANNEXES

8. Annexes

8.1. Annex A

Supplemental Material Chapter 2

Study on the digestion of milk with prebiotic carbohydrates in a simulated gastrointestinal model

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Table 1S. Composition of simulated salivary fluid (SSF)

Constituent	SSF (pH 7) /mmol/L)
K^+	18.8
Na^+	13.6
Cl^-	19.5
$H_2PO_4^-$	3.7
HCO_3^-, CO_3^{2-}	13.7
Mg^{2+}	0.15
NH_4^+	0.12
Ca^{2+}	1.2

α-amilase at 150 units per mL of SSF (Verhoeckx et al., 2015)

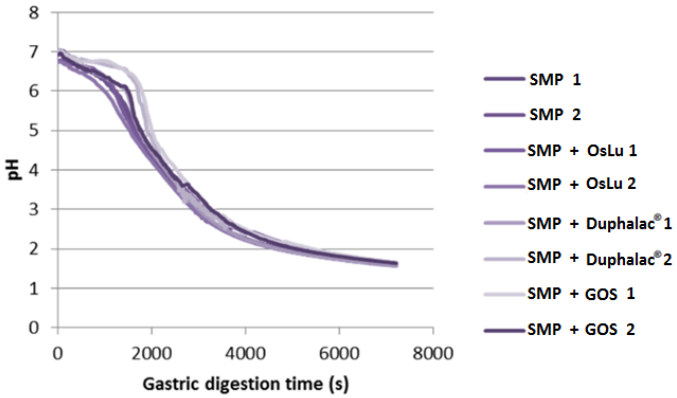

Figure 1S. pH profile of milk samples with the prebiotic ingredients during gastric digestion.

Table 2S. Carbohydrate composition (% of total carbohydrates) of OsLu, Vivinal®GOS and Duphalac®.

Samples	Glucose	Fructose	Galactose	Other Disaccharides	Lactose	Lactulose	Trisaccharides	Tetrasaccharides	Pentasaccharides	Hexasaccharides
OsLu	-	-	14.1 (1.0)	21.1 (1.1)	N.D.	26.1 (1.2)	25.6 (0.7)	9.7 (0.7)	2.6 (0.6)	0.2 (0.1)
Vivinal®GOS	20.7 (2.1)	-	1.4 (0.1)	20.5 (0.6)	18.0 (0.2)	-	21.0 (0.7)	13.1 (0.8)	4.8 (0.6)	0.7 (0.4)
Duphalac®	0.3 (0.0)	-	7.9 (0.7)	-	3.2 (0.2)	88.7 (0.6)	-	-	-	-

Data are expressed as the mean (SD) ($p>0.05$).
N.D. No detected.

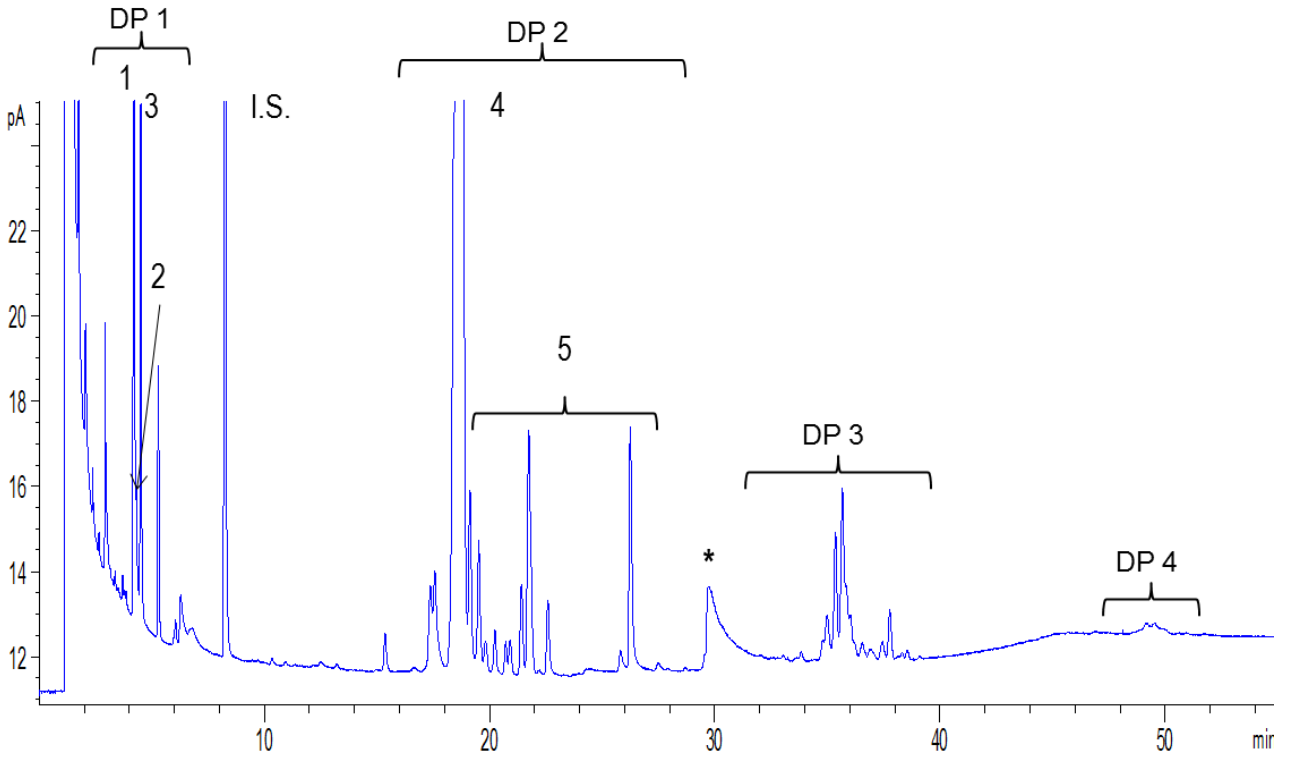

Figure 2S. GC-FID profile of TMSO derivatives of carbohydrates present in milk samples with OsLu after 1 h of gastric digestion. Peak 1 Galactose; 2 Glucose; 3 Galactose + Glucose; I.S. Internal Standard; 4 Lactose; 5 Other disaccharides. * Matrix effect, DP: Degree of Polymerisation.

8.2. Annex B

Supplemental Material Chapter 3

In vitro digestibility of galactooligosaccharides: Effect of the structural features on their intestinal degradation

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Table 1S. Hydrolysis degree (%) of di-, tri, oligosaccharides and lactose, lactulose in all samples during digestion with BBMV.

Σ Disaccharides					
Digestion time (min)		GOS-1	GOS-2	GOS-3	OsLu
	0	0	0	0	0
	1	13.8	0.0	13.3	2.4
	2	18.3	0.0	15.9	10.1
	3	22.8	0.0	19.7	13.2
	4	33.0	0.0	25.0	19.1
	5	43.7	0.0	32.6	21.5
Σ Trisaccharides					
Digestion time (min)		GOS-1	GOS-2	GOS-3	OsLu
	0	0	0	0	0
	1	17.4	24.7	3.0	6.4
	2	29.2	29.8	8.5	11.3
	3	44.0	37.1	9.2	12.8
	4	46.5	45.3	22.0	19.1
	5	54.6	50.2	24.1	25.5
Σ Oligosaccharides					
Digestion time (min)		GOS-1	GOS-2*	GOS-3	OsLu
	0	0	0	0	0
	1	15.7	15.1	6.6	3.9
	2	24.7	19.5	10.9	10.5
	3	34.9	26.4	12.9	13.2
	4	41.0	31.2	23.0	19.0
	5	50.1	34.9	27.1	22.8

Lactose/Lactulose		Lactose	Lactose	Lactose	Lactulose		
Digestion time (min)		GOS-1	GOS-2	GOS-3	OsLu	Lactose	Lactulose
	0	0	0	0	0	0	0
	1	27.2	5.6	19.9	10.8	42.9	6.3
	2	30.1	11.1	35.9	18.1	65.4	12.4
	3	39.2	27.8	53.8	23.9	69.5	17.3
	4	49.7	33.3	61.0	27.6	81.4	23.9
	5	68.7	50.0	68.5	32.9	97.1	29.6

*Represents the sum of di-, tri and tetrasaccharide (GOS-2)

8.3. Annex C

Supplemental Material Chapter 5

Behaviour of citrus pectin during its gastrointestinal digestion and fermentation in a dynamic simulator (simgi®)

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Table S1.

Physico-chemical overall characterization of citrus pectin. (Adapted from Muñoz-Almagro et al (2018) and Ferreira-Lazarte et al. (2018)

Humidity (%)	9.8 ± 0.6
a_w	0.22
pH	3.04 ± 0.01
Proteins (%)	0.67 ± 0.03
Minerals (mg g^{-1})	
Sodium	2.36
Magnesium	0.32
Potassium	0.60
Calcium	4.15
Furosine (mg 100 g^{-1} protein)	782.1 ± 5.2
Average M_w (kDa)	350
DM (%)	70.7
GalA content (%)	66.5 %

Table S2

Average plate count measurements (n=3) expressed in log CFU/mL, analysed in the ascending (AC), transverse (TC) and descending colon (DC) of the dynamic gastrointestinal model (simgi®) at the end of stabilization period (day 13), chronic intake (day 27) and washout period (day 34) with citrus pectin.

		Sampling Day		
Bacterial group		**Day 13 Stabilisation period**	**Day 27 Feeding period**	**Day 34 Washout period**
Total aerobes	AC	7.56 (0.01)^{b,1}	8.04 (0.12)^{c,2}	7.26 (0.13)^{a}
	TC	7.57 (0.11)^{c,1}	7.52 (0.08)^{b,1}	7.24 (0.12)^{a}
	DC	7.37 (0.01)^{b}	6.86 (0.03)^{a}	6.93 (0.22)^{a}
Total anaerobes	AC	8.26 (0.07)^{a}	8.50 (0.02)^{ab,2}	8.59 (0.08)^{b,1}
	TC	8.38 (0.04)^{b}	7.96 (0.07)^{a,1}	8.10 (0.14)^{ab}
	DC	8.30 (0.11)^{c}	7.35 (0.02)^{a}	7.95 (0.10)^{b}
Lactic bacteria	AC	5.76 (0.10)^{b}	6.42 (0.14)^{c,1}	4.59 (0.02)^{a}
	TC	6.30 (0.06)^{b,3}	6.63 (0.12)^{b,2}	4.57 (0.15)^{a}
	DC	5.98 (0.23)^{c,2}	5.81 (0.04)^{b}	5.08 (0.05)^{a,1}
Enterobacteria	AC	7.79 (0.05)^{b,1}	7.90 (0.03)^{b,2}	7.26 (0.23)^{a,1}
	TC	7.67 (0.13)^{b,1}	7.48 (0.12)^{b,1}	7.22 (0.02)^{a,1}
	DC	7.37 (0.28)^{b}	6.88 (0.03)^{a}	6.69 (0.02)^{a}
Enterococcos	AC	5.90 (0.05)^{a}	7.77 (0.07)^{c,2}	6.91 (0.24)^{b,1}
	TC	6.42 (0.12)^{c,1}	6.29 (0.09)^{bc}	5.37 (0.23)^{a}
	DC	6.03 (0.03)^{a}	7.50 (0.04)^{c,1}	6.58 (0.04)^{b,1}
C. perfringens	AC	7.55 (0.09)^{a,1}	7.51 (0.01)^{a,1}	7.45 (0.03)^{a,2}
	TC	7.63 (0.07)^{b,1}	7.75 (0.05)^{b,2}	6.19 (0.06)^{a}
	DC	7.22 (0.02)^{b}	7.22 (0.09)^{b}	6.52 (0.06)^{a,1}
Lactobacillus	AC	6.14 (0.11)^{c,1}	3.78 (0.07)^{b}	2.97 (0.14)^{a}
	TC	6.15 (0.07)^{b,1}	4.67 (0.09)^{b,1}	3.56 (0.08)^{a,1}
	DC	5.59 (0.03)^{c}	5.14 (0.05)^{b,2}	3.95 (0.10)^{a,2}

^{a,b,c} Different letters mean significant differences ($p < 0.05$) between the end of stabilisation (Day 13), feeding (Day 24-27) and washout period (Day 34) on the same compartment.

^{1,2,3} Different numbers mean significant differences ($p < 0.05$) between compartments on the same day of analysis. Standard deviation values are in brackets.

8.4. Annex D

Additional published scientific paper:

Application of a commercial digestive supplement formulated with enzymes and probiotics in lactase non-persistence management

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Food & Function
2018, 9, 4642-4650
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Abstract:

Strategies to avoid lactose malabsorption, which affects 70% of the world's population, are focused on the restriction of milk and dairy products or the use of non-human β-galactosidases or probiotics endowed with β-galactosidase activity added at mealtime. The evaluation of a commercial blend of probiotics and enzymes (protease, lactase, lipase and amylase) and its potential application in lactase non-persistence management are described in this work. Recommended amounts (460-1000 mg) of commercial probiotics/enzymes blend showed to be adequate for in vitro lactose hydrolysis in standard solutions (0.25-5 %) and commercial dairy products, milk (5% lactose) and yogurts (3% lactose) reaching hydrolysis values between 44-96%. According to these percentages, the use of the enzymatic preparation would guarantee the intake of less than 12 g, recommendations of the EFSA for lactose intolerant. Furthermore, formation of prebiotic galactooligosaccharides was also detected increasing the potential benefits of the enzymatic preparation in the gastrointestinal system.

1. Introduction

Over the past years, the interest toward human well-being and disease prevention has increased the consumption of healthy diets and a prudent addition of dietetic supplements,[1] being the gastrointestinal function one of the main targets. Particularly, the digestion and absorption of carbohydrates has been one of the main topics widely studied during the last years. Digestible di-, oligo - and polysaccharides are hydrolysed to their corresponding monomers before being absorbed in the small intestine; however, in some specific physiological or pathological situations, as in the case of lactose intolerance, these carbohydrates are hardly hydrolysed and absorbed, reaching the gut lumen where they are fermented by the intestinal microbiota.[2]

Lactose intolerance is a common problem resulting from β-galactosidase (i.e., lactase) deficiency at the level of the small intestine. With the rare exception of congenital hypolactasia, this enzyme is always present in the new-born, but its activity naturally diminishes after weaning. In Caucasians, a specific mutation favoured by the high intake of milk, permits the presence of lactase also in adults. However, in other geographical area these changes are not common and, approximately, a 70% of the world's population have non-

persistence of lactase. Particularly, in some Asian countries this rate increases up to 100%.[2,3]

Although lactose intolerance is not considered as a true ailment, its symptoms (abdominal spasms, swelling, flatulence and diarrhoea, with a considerable intraindividual and interindividual variability in the severity) may disturb the quality of life. The strategies to avoid this problem are mainly focused on the restriction of milk and dairy products and the intake of lactose-depleted and lactose-free products, non-exempt of nutritional and/or technical problems.[4] In this sense, some published studies have shown that thermal processing could involve a strong advance of the Maillard reaction (MR), with loss of available lysine and modifications in the sensorial properties when lactase is added before the heat treatment. As after hydrolysis the increase in galactose and glucose can greatly favour the evolution of this reaction, the addition of the enzyme after thermal processing is recommended; however, this involves the use of aseptic conditions with the consequent increase in the price of the products.[5,6] Moreover, in pack addition of lactase after milk sterilisation can have adverse organoleptic and nutritional concerns related to the enzyme side proteolytic activity especially for extended storage time.[7]

The intake of commercially lactase enzyme preparations in solid from fungal or yeast origin before lactose consumption has been also suggested as a

possibility for people with these problems. Although there are interesting studies that underline their potential applications, its usefulness is not fully established due to technical and dose discordances. In spite of this, the EFSA Panel on Dietetic Products, Nutrition and Allergies concluded that there is a cause-effect relationship between their consumption and breaking down lactose in individuals with symptomatic lactose malabsorption.[8] The administration of probiotics endowed with a lactase activity has been also showed to be very useful to treat patients with this problem.[3]

Recently, a new commercial product formulated with enzymes (protease, lactase, lipase and amylase) and non-dairy, heat-stable and stomach acid resistant probiotics (*Lactobacillus gasseri, Bifidobacterium bifidum* and *Bifidobacterium longum*) is offered as a supplement to support healthy digestive function and help alleviate occasional gas and bloating. The same supplement without enzymes has been proved to have anti-inflamatory effect due to the changes in the gut microbiota communities. An intervention study reported a higher percentage of participants who had an increase in bifidobacteria and lactobacillus in their faecal samples during the probiotic intervention versus the placebo.[9] However, to the best of our knowledge, no studies on the potential application of this preparation on malabsorption of carbohydrates have been carried out. Thus, the objectives of this work have

been: i) to characterise the carbohydrase activity of the commercial preparation of probiotics with enzymes and ii) to evaluate its *in vitro* effectiveness during the hydrolysis of lactose in lactose solutions and commercial dairy products such as whole milk, skimmed milk and two different yogurts.

2. Materials and methods

2.1.Chemicals and reagents

Fructose (Fru) standard was purchased from Fluka analytical™. D-galactose (Gal), D-glucose (Glc), lactose, sucrose, phenyl-β-glucoside, o-nitrophenyl (o-NP), p-nitrophenyl (p-NP), o-nitrophenyl-β-D-glucopyranoside (o-NPG) and p-nitrophenyl-α-glucopyranoside (p-NPG) standards were obtained from Sigma-Aldrich (St Louis, MO). Commercial whole and skimmed milk and yogurt samples were purchased from local markets in Madrid, Spain. Commercial enzymatic preparation (Kyo-Dophilus® plus enzymes) (human strains of *Lactobacillus gasseri* KS-13, *Bifidobacterium bifidum* G9-1 and *Bifidobacterium longum* MM2 together with protease (*Aspergillus melleus*), lactase (*A. oryzae*), lipase (*A. niger*) and amylase (*A. oryzae*)) was kindly supplied by Vitae® Natural Nutrition S. L. (Barcelona, Spain).

2.2.Characterisation of commercial preparation

Commercial enzymatic preparation was used to prepare an enzymatic solution according to the method of Olaokun, *et al*,[10] with minor modifications. Probiotics plus enzymes (10 mg/mL) was homogenized in ice-cold 0.05 M sodium phosphate buffer solution (pH 7.0, 6.5 and 6.5). Then, the clear solution was used for determining protein content, enzymatic activity and subsequent analysis by HPLC-ELSD and GC-FID. Furthermore, pH, water activity and dry matter analysis were carried out directly on the enzymatic preparation.

2.2.1. Physico-chemical characterisation

The dry matter content was gravimetrically determined in an oven at 110 °C during 48 hours until constant weight according to the Association of Official Analytical Chemists (AOAC).[11] Water activity (a_w) measurement was carried out in an AW Sprint TH-500 instrument (Novasina, Lachen, Switzerland). The pH of enzymatic preparation (1%, w/v) was obtained using a pHmeter (Mettler Toledo GmBH, Schwerzenbach, Switzerland). Protein content in enzymatic solution was determined by the Kjeldahl method as described by AOAC.[12]

2.2.2. Carbohydrase characterisation

The determination of β-galactosidase or lactase activity was adapted from Warmerdam *et al.*[13] A solution of o-NPG (o-nitrophenyl-β-D-glucopyranoside) in phosphate buffer 0.05 M, (pH 7.0, 6.5 and 6.0) with a concentration of 0.5 mg/mL (0.05% w/v) was prepared. Enzymatic activity was determined by incubating 1,900 µL of the o-NPG solution and 100 µL of enzyme solution from this commercial product (10 mg/mL in phosphate buffer 0.05 M, pH 7.0, 6.5 and 6.0) for 2 h at 37 ºC. The method is based on the measuring of the continuous release of o-NP from o-NPG. Absorbance of released o-NP was measured at 420 nm every 20 s using a spectrophotometer (Specord® Plus, Analytik Jena) together with a temperature controller (Jumo dTRON 308, Jumo Instrument Co.). Considering the lactase content on the enzymatic preparation, specific enzymatic activity (U) was expressed in µmol min^{-1} g^{-1}, where one unit was defined as the amount of enzyme that produced 1 µmol of o-NP in one min of reaction (n = 6).

Similar procedure was used to determine the maltase activity but using a solution of p-NPG (p-nitrophenyl-α-glucopyranoside) in phosphate buffer 0.05 M, pH 7.0, 6.5 and 6.0 with (0.05% w/w) and monitoring the release of p-NP at 410 nm every 20 s (n = 4).

Invertase activity was determined following the method described by Ghazi *et al*,[14] with slight modifications. An individual solution of sucrose (1 mg/mL) in sodium phosphate buffer 0.05 M, pH 7.0 was used. 50 mL of this solution were incubated together with 50 mg of enzymatic preparation with enzymes at 37 ºC during 2 h. Aliquots were taken at different times (15, 30, 60, 90 and 120 min) and reaction was stopped on boiling water during 5 min. Sucrase activity was determine by monitoring sucrose hydrolysis and increase of fructose by GC-FID. The specific enzymatic activity (U) was expressed in µmol min^{-1} g^{-1}, where one unit was defined as the amount of enzyme that produced 1 µmol of reducing sugars in one min of reaction (*n* = 4).

2.3.*In vitro* digestion of buffered standard solutions of lactose

To determine the effectiveness of the commercial preparation in the hydrolysis of lactose, an *in vitro* digestion study was carried out under simulated physiological conditions. This is a first common approach to understand the digestion of functional ingredients.[15]

First of all, to evaluate the resistance of lactose to the enzymatic preparation, several assays of lactose digestion were carried out using different carbohydrate-enzymatic preparation ratios (**Table 1**). Lactose concentrations were chosen to cover most of the commercial lactose-content products, such

as milk, yogurt, cheese, and free-lactose products.[16,17] In regard of the enzymatic preparation, doses were chosen taking into account recommended prescription (2 capsules/920 mg per day). Thus, 460 mg (1 capsule), 1000 mg and 155 mg of preparation were tested with each lactose concentration. Reactions that presented similarity on the ratio preparation/lactose with other reaction were discarded.

Table 1. Ratios of lactose to the probiotic preparation with enzymes during digestion at pH 7.0 or 6.5 at 37 ºC for 2h.

	Lactose (g)	Commercial preparation (mg)	Final volume (mL)	Commercial preparation/lactose ratio (E:S)
Lactose 5 %	12.5	155	250	0.01
Reactions	12.5	460	250	0.04
	12.5	1000	250	0.08
Lactose 1.0 %	2.5	460	250	0.18
Reactions	2.5	1000	250	0.40
Lactose 0.25 %	0.63	460	250	0.74
Reactions	0.63	1000	250	1.60

Hence, assays aimed to determine the capability of enzymatic preparation to hydrolyse lactose at different concentrations. Thus, different solutions of lactose (5.0, 1.0 and 0.25 %, w/v) in phosphate buffer 0.05 M pH 7.0 and 6.5 were tested with the following doses of enzymatic preparation: 155, 460 and 1000 mg. Finally, 250 mL of solution of lactose (5.0, 1.0 and 0.25 %, w/v) were mixed with each dose of enzymatic preparation (155, 460 and 1000

mg). The mixture was incubated at 37 ºC (pH 7.0 and 6.5) under continuous agitation (400 rpm) for 2 h. Aliquots were taken at 15, 30, 60, 90 and 120 min of digestion and heated in boiling water for 5 min to stop the reaction. The digestion of lactose was monitored by analysis of the trimethyl silylated oximes (TMSO) of carbohydrates by GC-FID as described below.

In addition, a series of control samples, based on the incubation of enzymatic preparation without lactose during the same reaction times, were also analysed. Results showed a minor increase of monosaccharides, galactose and glucose as the digestion proceeded. These values were conveniently subtracted in order to avoid any overestimation of the monosaccharide fraction.

2.4.*In vitro* digestion of lactose commercial products

Since buffered standard solutions of lactose would be more prone to changes as they are not protected in a food medium, the effectiveness of enzymatic preparation on lactose commercial products (two commercial milk and two yogurts) was tested. First, commercial products were characterised (**Table 2**) by measuring its pH, protein content, total carbohydrates and lactose content by GC-FID. Later, 250 mL of commercial milk and yogurt were mixed with 1000 mg of the commercial preparation of probiotics with enzymes and the mixture was incubated at 37 ºC (pH of milk and yogurt), 400 rpm for 2 h.

Aliquots were taken at 15, 30, 60, 90 and 120 min of digestion and the reaction was stopped by heating samples in boiling water for 5 min.

Before chromatographic analysis, samples were subjected to a clarification procedure using Carrez reagents in order to remove interfering compounds 6. Carbohydrates analysis was performed by GC-FID as described below.

2.5.Carbohydrate analysis by GC-FID

Trimethyl silylated oximes (TMSO) of carbohydrates (mono-, di- and trisaccharides) present in samples were determined following the method of Cardelle-Cobas.[18] Chromatographic analysis was carried out on an Agilent Technologies gas chromatograph (Mod7890A) equipped with a flame ionization detector (FID). The TMSO were separated using a 15 m x 0.32 mm x 0.10 µm film, fused silica capillary column (DB-5HT, J&W Scientific, Folson, California, USA). Nitrogen was used as carrier gas at a flow rate of 1 mL/min. Injector and detector temperatures were 280 and 385 ˚C, respectively. The oven temperature was programmed from 150 to 380 ˚C at a heating ratio of 3 ˚C/min. Injections were made in the split mode (1:20 or 1:5) depending on lactose content of the solution.

Table 2. Physico-chemical characteristics of lactose commercial products.

Product	Carbohydrate content (%)	Lactose content (%)	pH	Protein content (%)	Theoretical Fat content (%)
Whole milk	4.9 ± 0.1	4.9 ± 0.1	6.9	3.0	3.6
Skimmed milk	5.1 ± 0.2	5.1 ± 0.2	6.9	3.1	0.3
Natural Yogurt	4.4 ± 0.2	3.6 ± 0.2	4.5	3.9	0.1
Liquid Yogurt	4.0 ± 0.2	3.2 ± 0.2	4.5	3.1	0.5

The TMSO derivatives were formed following the method of Ruiz-Matute *et al.*[6] First, a volume of 100 or 200 µL of the digested sample was added to 400 µL of internal standard solution, containing 0.5 mg/mL of phenyl-β-glucoside. Afterwards, the mixture was dried at 40 ˚C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). Sugar oximes were formed by adding 250 µL hydroxylamine chloride (2.5%) in pyridine and heating the mixture at 70 ˚C for 30 min. Subsequently, the oximes obtained in this step were silylated with hexamethyldisylazane (250 µL) and trifluoroacetic acid (25 µL) at 50 ˚C for 30 min.[19] Derivatization mixtures were centrifuged at 6,700 x *g* for 2 min and supernatants were injected in the GC.

Data acquisition and integration were done using Agilent ChemStations software (Wilmington, DE, USA). Response factors were calculated after the duplicate analysis of standard solutions (fructose, galactose, glucose, lactose, sucrose, raffinose and stachyose), at different concentrations ranging from 0.005 to 4 mg/mL.

2.6. Statistics

All digestions were carried out in duplicate and two GC-FID analysis were carried out for each digestion treatment (n = 4). The comparisons of means using analysis of variance (ANOVA) were made using the statistical package (SPSS Inc., Chicago, Il). The differences were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Characterisation of the commercial preparation of probiotics with enzymes

The overall characterisation of the enzymatic preparation showed that the pH was 6.46, similar to that of small intestine in adults and children, where the hydrolysis of carbohydrates takes places.[15,20] The a_w (0.186) and dry matter (94.6%) values guarantee its microbiological stability. Regarding protein, data obtained by the Kjeldhal method were slightly higher (17.4%) than the sum of the amounts of all enzymes reported in the product (for 1 capsule of 460 mg: 35 mg protease, 17.5 mg lactase, 12.5 mg lipase y 12.5 mg amylase; total 77.5 mg, 16.8%). This small difference could be due to the different methods used and the presence of proteins coming from the probiotic bacteria.

The next step was the evaluation of the main carbohydrase activities in the enzymatic preparation, being lactase, maltase and invertase the tested activities as indicated in Material and Methods. Although, according to the data sheet, the commercial preparation presented other enzymatic activities different from those related to carbohydrates, they were not evaluated. This was out from the aim of the work and, as indicated earlier, nowadays a lot of attention is paid to the carbohydrate malabsorption. **Figure 1** shows the evolution of the corresponding activities carried out at pH 6, 6.5 and 7. Maltase (B) and invertase (C) followed a similar behaviour with a constant increase through the time; however, lactase (A) had a different pattern with a first lineal phase to reach a plateau after 10 min at pH 7 and 5 min at pH 6.5 and 6. In lactase and maltase the highest activity was detected at pH 7. Taking into account the data of **Figure 1**, the specific enzymatic activities were calculated (**Table 3**), the highest activity being lactase, followed by maltase and invertase. Cardelle-Cobas, (2009)[18] tested an enzymatic preparation from the same source (*Aspergillus oryzae*) and found a β-galactosidase activity of ~7000 U/g. Taking into account these results, the main objective of this work was focused on the usefulness of the commercial enzymatic preparation on lactose hydrolysis, in order to broaden its applicability.

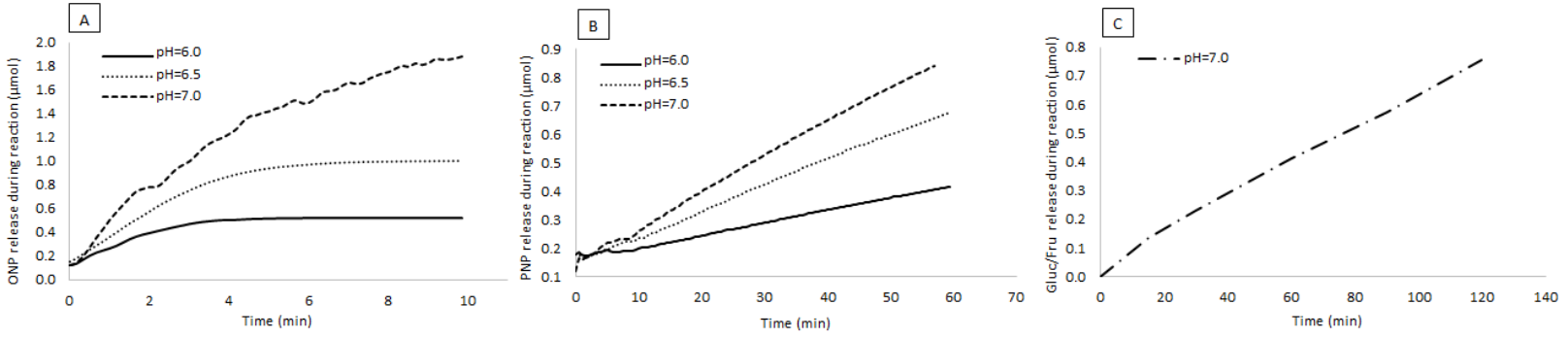

Figure 1. Release rate of compounds during enzymatic activity measurement of the commercial preparation (CP) (µmol). A) *o*-NP release, β-galactosidase activity. B) *p*-NP release, maltase activity. C) Glucose and Fructose release, Invertase activity.

Table 3 Enzymatic activities of the commercial preparation measured at the studied conditions.

Activity	Substrate	U (μmol/min*g)		
		pH = 7.0	pH = 6.5	pH = 6.0
β-Galactosidase	*o-NPG*	6815.9 ± 119.5	5006.3 ± 29.0	3342.4 ± 39.5
Maltase	*p-NPG*	487.3 ± 14.4	290.7 ± 23.4	132.8 ± 12.7
Invertase	Sucrose	39.2 ± 3.6	-	-

3.2. Hydrolysis of lactose in buffered standard solutions

Figure 2 depicts the chromatograms obtained by GC-FID of the hydrolysis of lactose (A, 5%; B, 1%; C, 0.25%) after 2 h of digestion with 1000 mg of commercial preparation. Galactose and glucose were formed together with different di- and trisaccharides derived from the transgalactosylation of lactose. It has been previously described that β-galactosidase can hydrolyse or transgalactosylate lactose forming molecules of higher molecular mass depending on the reaction conditions.[21,22] As lactases from *A. oryzae* synthesise galactooligosaccharides (GOS) prebiotic with β(1-6) linkages it is plausible that the structures formed during the *in vitro* digestion of lactose with the enzymatic preparation are prebiotics. In addition to the action of lactase derived from *A. oryzae*, it is also presumable that the probiotic bacteria (bifidobacteria and lactobacilli) also present in the supplement contribute to these reactions.

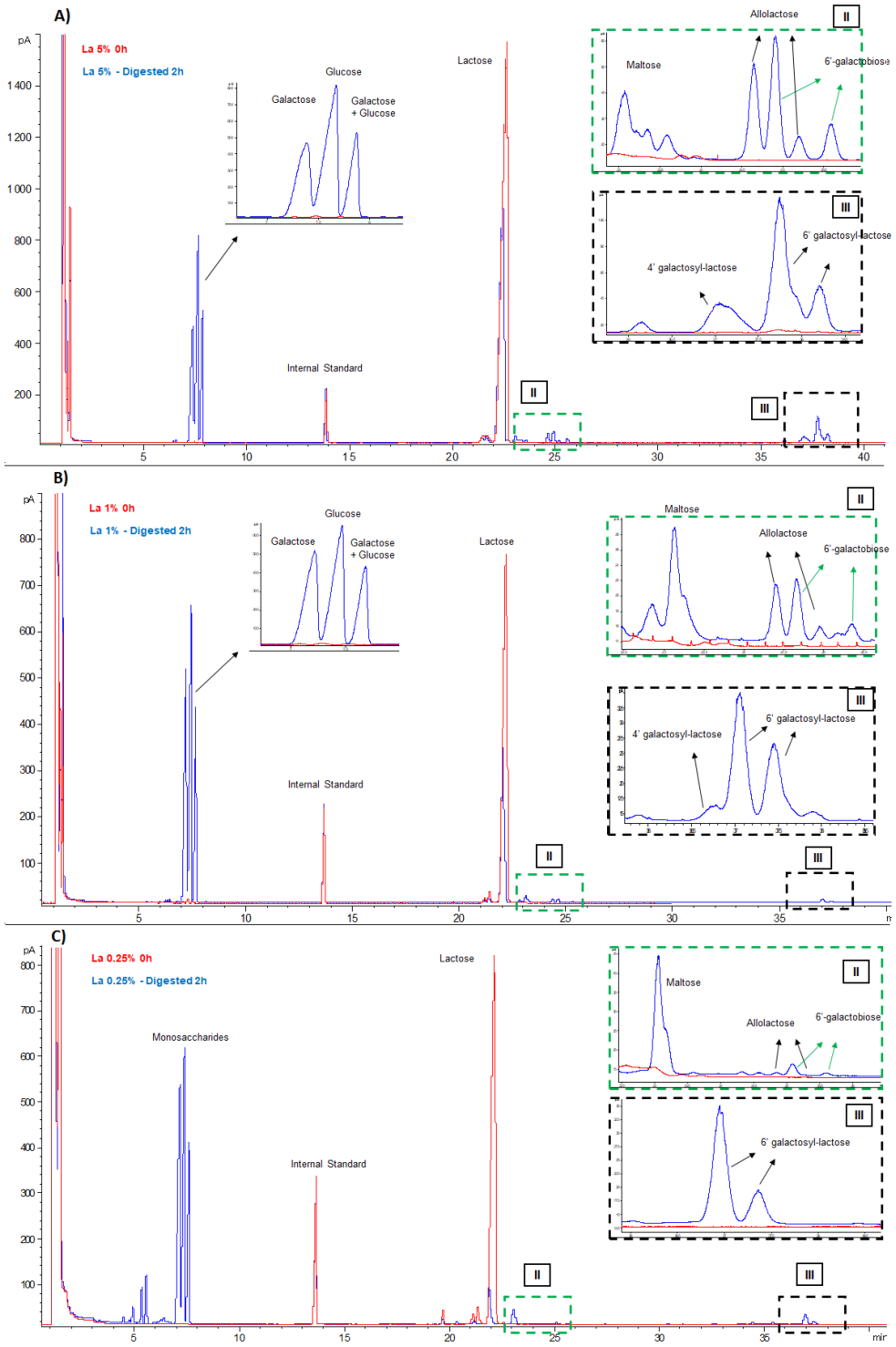

Figure 2. Chromatographic profiles obtained by GC-FID corresponding to the hydrolysis of lactose (A, 5%; B, 1%; C, 0.25%) with the commercial preparation (1000 mg) at 37 ˚C, pH 7.0, at 0 h of reaction (blank**, red**) and 2 h of reaction (**blue**).

In this sense, β-galactosidases derived from lactic acid bacteria and bifidobacteria are also of valuable interest for production of GOS with better selectivity for the growth and metabolic activity of these two bacteria genera in the gut, which may lead to an improved prebiotic effect.[23]

Tables 4 and 5 show the results obtained after all the reactions carried out with lactose solutions at pH 7 and 6.5, taking into account the ratios commercial preparation/lactose (w/w) indicated in *Materials and Methods* (**Table 1**). As expected, lactose hydrolysis increased with the increase of preparation and with the decrease of lactose concentration. In general, the highest hydrolysis was found in the reactions performed at the lowest pH. At pH 6.5, three reactions led to percentage values of hydrolysis higher than 90%, and in one of them was almost 99%, whereas at pH 7 only in one reaction the hydrolysis value exceeded 90% (maximum amount of enzyme, 1000 mg, and minimum of lactose, 0.25%). However, the β-galactosidase activity carried out with o-NPG above mentioned, was higher at pH 7 than at pH 6.5. These dissimilarities could be ascribed to different selectivity of enzymes (from *A. oryzae* and probiotics bacteria) toward substrates, o-NPG and lactose.

Table 4 Lactose hydrolysis (%) during the treatment of different solutions of lactose with enzymatic preparation at pH 7.0.

Time (min)	CP 155* - La5%	CP 460 - La5%	CP 1000 - La5%	CP 460 - La1%	CP 1000 - La1%	CP 460 - La0.25%	CP 1000 - La0.25%
0	-	-	-	-	-	-	-
15	8.0 ± 0.9^{a}	21.5 ± 1.2^{b}	22.0 ± 0.8^{b}	24.6 ± 0.1^{b}	25.9 ± 0.9^{b}	32.9 ± 0.9^{c}	44.4 ± 0.8^{d}
30	14.1 ± 1.1^{a}	25.8 ± 0.9^{b}	29.2 ± 1.1^{b}	33.0 ± 0.5^{b}	$37.3 \pm 1.1^{b.c}$	45.2 ± 0.7^{c}	57.7 ± 0.6^{d}
60	19.8 ± 0.6^{a}	35.0 ± 1.1^{b}	40.4 ± 0.4^{c}	43.8 ± 0.3^{d}	52.6 ± 1.6^{e}	64.2 ± 0.5^{f}	80.6 ± 1.5^{g}
90	22.8 ± 0.7^{a}	39.2 ± 1.2^{b}	45.4 ± 0.3^{c}	50.7 ± 0.4^{d}	61.7 ± 0.3^{e}	77.2 ± 0.7^{f}	89.4 ± 1.1^{g}
120	26.8 ± 1.2^{a}	44.0 ± 0.8^{b}	50.9 ± 0.5^{c}	57.6 ± 0.7^{d}	68.8 ± 1.0^{e}	80.6 ± 0.8^{f}	93.1 ± 1.2^{g}

Data are presented as mean ± SD (n = 4).

*Represents the amount of commercial preparation used (mg).

$^{a, b, c}$ Different letters indicate statistical differences in lactose hydrolysis (%) between all tested samples at the same reaction time using a one-way analysis of variance (ANOVA) ($p < 0.05$) (n=4).

Table 5 Lactose hydrolysis (%) during the treatment of different solutions of lactose with enzymatic preparation at pH 6.5.

Time (min)	CP 155* - La5%	CP 460 - La5%	CP 1000 - La5%	CP 460 - La1%	CP 1000 - La1%	CP 460 - La0.25%	CP 1000 - La0.25%
0	-	-	-	-	-	-	-
15	15.5 ± 1.6^{a}	28.1 ± 1.1^{b}	34.2 ± 0.8^{b}	34.9 ± 0.2^{b}	49.1 ± 0.1^{c}	47.6 ± 1.9^{c}	68.7 ± 0.9^{d}
30	21.7 ± 0.9^{a}	32.0 ± 0.8^{b}	45.8 ± 1.1^{c}	48.9 ± 0.9^{c}	67.5 ± 0.9^{d}	70.7 ± 1.0^{d}	85.7 ± 1.2^{e}
60	31.3 ± 0.6^{a}	42.0 ± 0.6^{b}	56.5 ± 1.2^{c}	66.4 ± 1.0^{d}	81.9 ± 1.2^{e}	86.5 ± 0.5^{f}	95.3 ± 1.4^{g}
90	37.0 ± 0.4^{a}	49.2 ± 1.0^{b}	62.3 ± 0.9^{c}	73.1 ± 0.9^{d}	90.2 ± 1.6^{e}	93.7 ± 1.8^{e}	98.5 ± 1.6^{f}
120	41.0 ± 0.9^{a}	54.0 ± 0.8^{b}	68.3 ± 0.7^{c}	78.3 ± 1.8^{d}	91.6 ± 1.8^{e}	96.6 ± 1.6^{f}	99.3 ± 1.2^{f}

Data are presented as mean ± SD (n = 4).

* Represents the amount of commercial preparation used (mg).

$^{a, b, c}$ Different letters indicate statistical differences in lactose hydrolysis (%) between all tested samples at the same reaction time using a one-way analysis of variance (ANOVA) ($p < 0.05$) (n=4).

Figure 3 shows the evolution at pH 7 (A) and 6.5 (B) of GOS (di- and trisaccharides) formed during the corresponding reactions of lactose hydrolysis. At both pH values, the highest GOS content was detected at the maximum lactose concentration (5%) since at lower concentration of substrate the transgalactosylation is not favoured and the GOS formed are hydrolysed rapidly by the enzymes.[22] Values of GOS concentrations in the range 30-120 mg/g lactose were found.

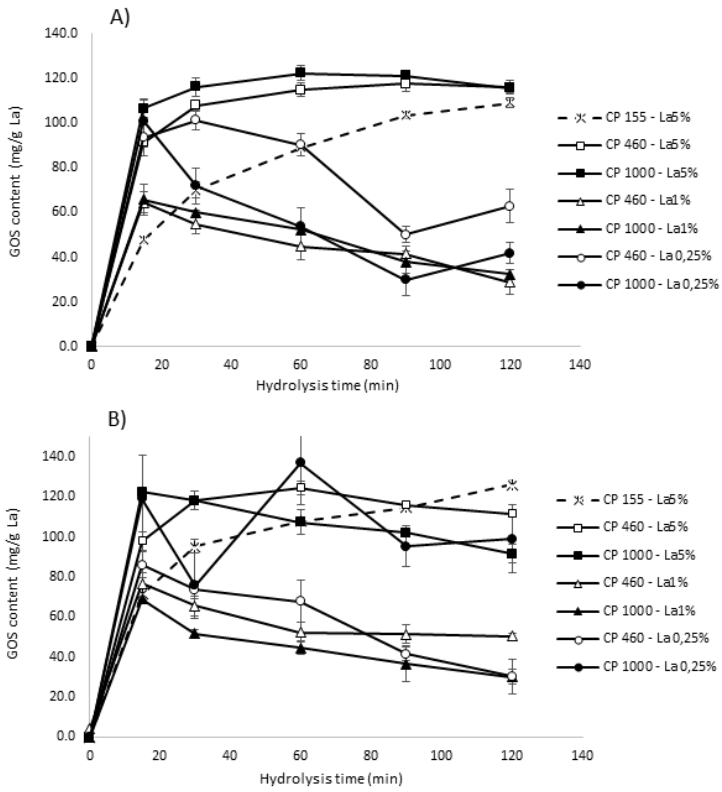

Figure 3. Evolution of the Galactooligosaccharides (GOS) content during the treatment with the commercial preparation (CP) of probiotics and enzymes at 37 ˚C, for 2 h and pH 7.0 (A) and 6.5 (B).

The evolution of the hydrolysis of lactose in these products is revealed in **Figure 4**. It is clear that both types of products had a different behaviour against the enzymatic preparation with a higher hydrolysis in yogurts (>91%) than in milk (>55%), probably due to the lower amount of initial lactose in yogurts and/or the lower pH, since the maximum activity of lactase from *A. oryzae* takes places at pH values of 2.5-5.5. In addition, the presence of lactase coming from the live starter cultures could also contribute to the lactose hydrolysis.[24]

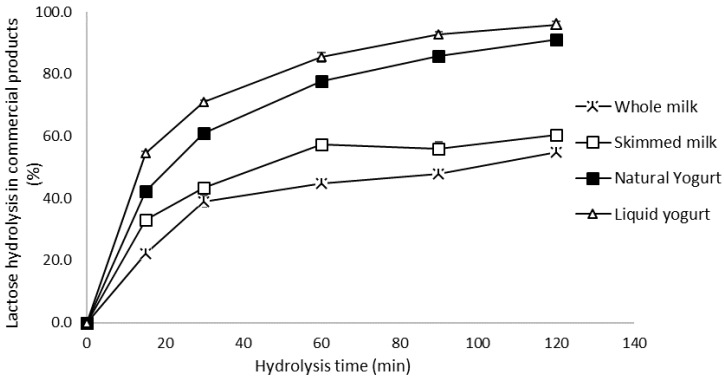

Figure 4. Hydrolysis of lactose (%) in commercial products (milk and yogurt) during the treatment with commercial preparation (1000 mg) at 37 ˚C for 2 h.

When whole and skimmed milks were compared, the hydrolysis occurred in significant less extent in the former (54.8%) than in the latter (60.4%). These values are slightly lower than those obtained during the hydrolysis of lactose

in buffered standard solutions under the same conditions which gave rise to a value of 69.3%. Therefore, a protection effect of milk composition against the hydrolysis of lactose with lactase from *A. oryzae* was observed. The upper thickness and, therefore, reduced distribution rates of both the enzyme and the substrate, in addition to possible hydrophobic binding by fat globules in whole milk, are likely reasons of these data.[25]

Similarly to the results with lactose solutions, there was higher formation of GOS (**Table 6**) in the commercial products with lower hydrolysis (milks, around 5,500 mg/L) and this amount was almost kept as a plateau during all the digestion process and were much higher than the values of GOS found by Ruiz-Matute *et al*.[6] in commercial UHT milks (average, 2,134 mg/L). Later, Larsen et al (2015)[26] reported a patented method for preparing lactose-depleted and rich GOS products having a stable content of GOS using lactase from *B. bifidum*.

Table 6 GOS content evolution (mg/L) during the treatment of commercial dairy products with the enzymatic preparation Kyo-Dophilus® (1000 mg) with enzymes.

Time (min)	Kyo 1000 – WM (5%)	Kyo 1000 – SM (5%)	Kyo 1000 – NY (3.6%)	Kyo 1000 – LY (3.2%)
0	-	-	-	-
15	5162.8 (11.9 %)	5626.8 (12.7 %)	5385.6 (10.1 %)	2637.3 (6.4 %)
30	4603.6 (11.8 %)	5705.3 (13.2 %)	4496.0 (8.5 %)	2506.6 (5.8 %)
60	5099.5 (11.9 %)	4784.7 (12.3 %)	3450.5 (6.4 %)	1912.0 (4.1 %)
90	5216.2 (11.1 %)	5361.3 (11.5 %)	2634.8 (4.7 %)	1323.5 (2.9 %)
120	5069.3 (10.8 %)	5321.4 (11.1 %)	2196.0 (3.7 %)	1099.3 (2.3 %)

*() Represents the percentage of GOS in every sample.
WM, Whole Milk; SM, Skimmed Milk; NY; NY, Natural Yogurt; LY, Liquid Yogurt

3. Conclusions

The data found in this research allow us to conclude that the studied commercial supplement of enzymes and probiotics, in the quantities (≤1000 mg, two capsules) and conditions here assayed, is adequate for the hydrolysis of lactose in buffered solutions (0.25 - 5%) and in commercial dairy products, milk (5% lactose) and yogurts (3% lactose). Hydrolysis of lactose values ranged from 27 to 99%, depending on the relationship of enzyme preparation / lactose and the type of the product. The highest hydrolysis was found in lactose solutions followed by yogurts, and especially a protective effect of the matrix was also observed in whole milk. In this commercial product, considering the intake of 1000 mg, a hydrolysis higher than 55% was observed, which would guarantee the intake of less than 12 g, recommendations of the EFSA Panel on Dietetic Products, Nutrition and

Allergies for lactose intolerant 8. In addition, due to the transgalactosylation potential of lactases present in this commercial preparation under the conditions tested, prebiotic GOS are also formed, expanding the applications of probiotics plus enzymes. Although more research is needed, this preparation could be taken with meals to assist in the digestion of lactose or be also used to easily prepare lactose-depleted and enriched in GOS products before consumption.

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Conflict of interest

The authors have no competing personal or financial interests to declare.

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8.5. Annex E

Published scientific papers

Chapter 1: Assessment of *in vitro* digestibility of dietary carbohydrates using rat small intestinal extract

Chapter 2: Study on the digestion of milk with prebiotic carbohydrates in a simulated gastrointestinal model

Chapter 3: *In vitro* digestibility of galactooligosaccharides: Effect of the structural features on their intestinal degradation

Chapter 4: *In vitro* fermentation properties of pectins and enzymatic-modified pectins obtained from different renewable bioresources

Chapter 5: Behaviour of citrus pectin during its gastrointestinal digestion and fermentation in a dynamic simulator (simgi®)

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Article

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Assessment of *in Vitro* Digestibility of Dietary Carbohydrates Using Rat Small Intestinal Extract

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ABSTRACT: There are few studies on the assessment of digestibility of nondigestible carbohydrates, despite their increasingly important role in human health. *In vitro* digestibility of a range of dietary carbohydrates classified as digestible (maltose, sucrose, and lactose), well-recognized (lactulose, fructooligosaccharides (FOS), and two types of galactooligosaccharides (GOS) differing in the predominant glycosidic linkage), and potential (lactosucrose and GOS from lactulose, OsLu) prebiotics using a rat small intestinal extract (RSIE) under physiological conditions of temperature and pH is described. Recognized and potential prebiotics were highly resistant to RSIE digestion although partial hydrolysis at different extents was observed. FOS and lactulose were the most resistant to digestion, followed closely by OsLu and more distantly by both types of GOS and lactosucrose. In GOS, $\beta(1 \rightarrow 6)$ linkages were more resistant to digestion than $\beta(1 \rightarrow 4)$ bonds. The reported *in vitro* digestion model is a useful, simple, and cost-effective tool to evaluate the digestibility of dietary oligosaccharides.

KEYWORDS: *nondigestible oligosaccharides, prebiotics, in vitro digestion model, intestinal digestibility, mammalian digestive enzymes, carbohydrases*

INTRODUCTION

There is growing evidence indicating that dietary nondigestible oligosaccharides (NDO) play an increasingly important role in health. Low glycemic index foods, characterized by slowly absorbed carbohydrates, are linked with reduced risk of common chronic Western diseases associated with central obesity and insulin resistance.[1,2] These pieces of evidence have boosted the interest in the use of nondigestible (or with slow digestion rate) carbohydrates as food ingredients due to their ability to reduce postprandial glycemic response.[3] The attention to NDO is also reinforced by the fact that regulatory agencies such as the EFSA have acknowledged that the consumption of foods/drinks, in which NDO replace simple sugars, reduces postprandial glycaemic and insulinaemic responses. This behavior is attributed to the resistance of NDO to hydrolysis and absorption in the small intestine.[4−7]

A specific subset of nondigestible carbohydrates, so-called prebiotics, have attracted especial interest due to their capability to reach the colon and be selectively fermented by the intestinal microbiota that results in specific changes in its composition and/or activity, thus contributing to human health promotion.[8,9] Intestinal microbiota plays an important role in a great variety of physiological process, such as the development of the host immune system, anti-inflammatory activity, uptake of energy from the host diet, production of short-chain fatty acids by fermentation, alteration of human glucose and fatty acid metabolism, regulation of intestinal permeability, or stimulation of mineral absorption by the large intestine.[10−13]

However, despite the generally accepted concept that NDO pass through the upper gastrointestinal tract without substantial modifications,[9] few efforts have been made toward the study of the resistance of this type of oligosaccharides to the digestion in the small intestine, and only scarce and fragmented information on their pass throughout the small intestine is available. In this context, the limitations of AOAC method 2009.01[14] for the measurement of NDO have already been highlighted, such as the use of a very limited number of enzymes (i.e., α-amylase and amyloglucosidase) which fail to hydrolyze digestible saccharides (including sucrose or starch-decomposed products), as well as the use of enzymes from fungal origin despite it being well-known that the hydrolyzing activity of enzymes from fungal or microbial sources does not reflect the carbohydrase activities of enzymes of the human gastrointestinal tract.[15] Consequently, alternative methods which are based on the use of mammalian intestinal enzymes, such as those derived from pigs[16] and weaning piglets,[17] have recently been proposed. However, up to date, the regular supply of porcine small intestinal enzymes is not commercially available, which may hinder an easy and broad implementation of these useful methods to evaluate the *in vitro* intestinal digestion of oligosaccharides. In this sense, the use of rat small intestinal extract (RSIE) can be advantageous because of its commercial availability, as well as the reported similarity of hydrolyzing activities between human and rat small intestinal disaccharidases.[18] In fact, the use of RSIE has been successfully applied for the assessment of digestibility of prebiotics, such as fructooligosaccharides (FOS)[19] and galactooligosaccharides (GOS),[20,21] as well as to a range of maltose and sucrose isomers[3] and isomaltooligosaccharides.[22] However, to the best of our knowledge, a comparative study of well-recognized prebiotics, that is FOS, GOS, and lactulose, and potential and novel candidates, such as lactosucrose and GOS derived from lactulose (OsLu), has not been carried out. Particularly, GOS

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comprise a complex mixture of, mainly, disaccharides and trisaccharides having a variety of glycosidic linkages with β-anomeric configuration. While β(1 → 4) and β(1 → 6) are the most common glycosidic linkages found in GOS structures, β(1 → 2) and β(1 → 3) are quite rarely.[23,24] Bearing in mind that there is evidence in the literature indicating that the linkage type could be a factor more important than monomer composition in determining the susceptibility of carbohydrates to digestive glycosidases,[3] the enzymatic susceptibility of GOS could be largely affected by differences in the predominant glycosidic linkage.

Therefore, the aim of the present study was to evaluate the small intestinal digestibility of well-recognized prebiotics, that is lactulose, FOS (kestose and nystose), and two types of conventional GOS with predominant β(1 → 4) or β(1 → 6) linkages, respectively, as well as emerging prebiotic candidates such as lactosucrose and OsLu, and their comparison with digestible disaccharides (lactose, sucrose, and maltose) used as appropriate controls in an *in vitro* digestion model using RSIE.

MATERIALS AND METHODS

Chemicals and reagents. Fructose (Fru) standard was purchased from Fluka analytical. D-Galactose (Gal), D-glucose (Glc), lactose (β-D-Gal(1 → 4)-D-Glc), sucrose (β-D-Fru(2 → 1)-α-D-Glc), maltose (α-D-Glc(1 → 4)-D-Glc), trehalose (α-D-Glc(1 → 1)-α-D-Glc), palatinose (also termed isomaltulose) (α-D-Glc(1 → 6)-D-Fru), lactulose (β-D-Gal(1 → 4)-D-Fru), kestose (β-D-Fru(2 → 1)-β-D-Fru(2 → 1)-α-D-Glc), nystose (β-D-Fru(2 → 1)-β-D-Fru(2 → 1)-β-D-Fru(2 → 1)-α-D-Glc), phenyl-β-glucoside, *o*-nitrophenyl (*o*-NP), *p*-nitrophenyl (*p*-NP), *o*-nitrophenyl-β-D-glucopyranoside (*o*-NPG) and *p*-nitrophenyl-α-glucopyranoside (*p*-NPG) standards, and intestinal acetone powders from rat (Rat Small Intestinal Extract, RSIE) were obtained from Sigma-Aldrich (St Louis, MO). Lactosucrose (β-D-Gal(1 → 4)-α-D-Glc(1 → 2)-β-D-Fru) standard was obtained from Wako Chemical Industries (Neuss, Germany). All standard carbohydrates were of analytical grade (purity ≥95%).

Obtainment of prebiotic ingredients. OsLu were obtained at pilot scale by the company Innaves S.A. (Vigo, Spain) following the method described by López-Sanz et al.[25] In brief, OsLu were synthesized using a commercial lactulose preparation (670 g/L; Duphalac, Abbott Biologicals B.V., Olst, The Netherlands), diluted with water at 350 g/L and pH adjusted to 6.7 with KOH, and a β-galactosidase from *Aspergillus oryzae* (16 U/mL; Sigma). The mixture of oligosaccharides (20% [w/v]) was treated with fresh *Saccharomyces cerevisiae* (1.5% [w/v]; Levital, Paniberica de Levadura S.A., Valladolid, Spain) at 30 °C and aeration at 20 L/min to remove monosaccharides. Finally, the samples were vacuum concentrated at 40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). The two commercial GOS syrups with predominant β(1 → 4) (named GOS-1) and β(1 → 6) (named GOS-2) linkages were kindly provided by the corresponding manufacturers whereas a mixture of FOS consisting of kestose and nystose was obtained from Wako Chemical Industries (Neuss, Germany).

The composition of OsLu syrup, whose main involved glycosidic linkage was β(1 → 6), expressed in g per 100 g of ingredient was as follows: 0.5% fructose, 12.5% galactose, 26% lactulose, 19.6% OsLu disaccharides, 16.0% OsLu trisaccharides (making 61.6% of potential NDO) and 25.7% moisture. GOS-1 syrup had 21% moisture and the composition of carbohydrates was 1% galactose, 21.4% glucose, 13% lactose, 19.2% GOS-disaccharides, 20.8% GOS-trisaccharides and 3.6% GOS-tetrasaccharides (equivalent to 43.6% of potential NDO). GOS-2 syrup composition was: 6.7% galactose, 22.6% glucose, 19.0% lactose, 9.9% GOS-disaccharides, 14.2% GOS-trisaccharides, 0.7% GOS-tetrasaccharides (equivalent to 24.8% of potential NDO) and 27% moisture.

Determination of protein content and main enzyme activities of the Rat Small Intestinal Extract (RSIE). RSIE was used to prepare an enzyme/enzymatic solution according to the method of Olaokun et al.,[26] with minor modifications. RSIE (10 mg/mL) was homogenized in ice-cold 0.05 M sodium phosphate buffer solution. Then, the solution was centrifuged at 2,415 x *g* for 15 min and the supernatant obtained was used as the enzyme solution for determining protein content and enzymatic activity.

Protein content. The total protein content of the enzymatic solution was quantified according to the Bradford method[27] using the Bio-Rad Protein Assay kit and bovine serum albumin as a standard. The absorbance was monitored at 595 nm.

Hydrolytic activities. *β-galactosidase and maltase activities.* The determination of the rat intestinal β-galactosidase activity was adapted from Warmerdam et al.[28] A solution of *o*-NPG in phosphate buffer 0.05 M, pH 7.0 with a concentration of 0.5 mg/mL (0.05% w/w) was prepared. The enzymatic activity was determined by incubating 1,900 μL of the *o*-NPG solution and 100 μL of enzyme solution from RSIE for 2 h at 37 °C. The method is based on the measuring of the continuous release of *o*-NP from *o*-NPG. Absorbance of released *o*-NP was measured at 420 nm every 20 s using a spectrophotometer (Specord Plus, Analytik Jena) together with a temperature controller (Jumo dTRON 308, Jumo Instrument Co.). The specific enzymatic activity (U) was expressed in μmol min^{-1} g^{-1}, where one unit was defined as the amount of enzyme that produced 1 μmol of *o*-NP in one min of reaction (n = 6).

Similar procedure was used to determine the maltase activity but using a solution of *p*-NPG in phosphate buffer 0.05M, pH 6.8 with (0.05% w/w) and monitoring the release of *p*-NP at 420 nm every 20 s (n = 3).

Sucrase, trehalase and palatinase activities. Sucrase, trehalase and palatinase activities were determined following the method described by Ghazi et al.,[29] with slight modifications. An individual solution of sucrose, trehalose or palatinose (0.5% w/w) in sodium phosphate buffer 0.05 M, pH 6.5 was used. An eppendorf tube with 250 μL of sucrose, trehalose or palatinose solution was preheated at the reaction temperature, 37 °C. Subsequently, 100 μL of enzyme solution was added and the mixture was incubated for 2 h and different aliquots were taken at different times (5, 10, 15, 30, 60, 90, and 120 min). Hydrolysis was stopped by adding 350 μL of a 3,5-dinitrosalicylic acid (DNS) solution prepared according to Asare-Brown & Bullock.[30] Sucrase, trehalase and palatinase activities were determined measuring the reducing sugars released from the corresponding disaccharide hydrolysis, at 540 nm, according to the DNS method.[31] The specific enzymatic activity (U) was expressed in μmol min^{-1} g^{-1}, where one unit was defined as the amount of enzyme that produced 1 μmol of reducing sugars in one min of reaction (n = 3).

In vitro small intestinal digestion using RSIE. The digestibility of two types of conventional GOS, (GOS-1 and GOS-2), OsLu, a mixture of FOS (comprised of kestose and nystose), lactosucrose, lactulose and digestible oligosaccharides such as lactose, sucrose and maltose were evaluated using RSIE. In a first step, preliminary assays aimed to determine an optimal RSIE-carbohydrate weight ratio within 2 h of reaction were carried out using lactulose and lactose as appropriate controls. Finally, a solution of 20 mg of RSIE and 1 mL distilled water was prepared as a digestive enzyme solution, resulting in a pH value of 6.8. Subsequently, 0.5 mg of carbohydrate was added and the mixture was incubated at 37 °C under continuous agitation (450 rpm) for 2 h. Aliquots were taken at 15, 30, 60, 90, and 120 min of digestion and heated in boiling water for 5 min to stop the reaction. The digestion was monitored by GC-FID as described below.

In addition, a series of control samples, based on the incubation of RSIE without carbohydrates during the same reaction times, were analyzed. Results showed a slight increase of galactose and a notable release of glucose as the digestion proceeded. These values were conveniently subtracted in order to avoid any overestimation of the monosaccharide fraction.

Carbohydrate analysis by GC-FID. Trimethylsilylated oximes (TMSO) of carbohydrates (mono-, di- and trisaccharides) present in samples were determined following the method of Cardelle-Cobas et al.[32] Chromatographic analysis was carried out on an Agilent

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Technologies gas chromatograph (Mod7890A) equipped with a flame ionization detector (FID). The TMSO were separated using a 15 m x 0.32 mm × 0.10 μm film, fused silica capillary column (DB-5HT, J&W Scientific, Folson, California, USA). Nitrogen was used as carrier gas at a flow rate of 1 mL/min. Injector and detector temperatures were 280 and 385 °C, respectively. The oven temperature was programmed from 150 to 380 °C at a heating ratio of 3 °C/min. Injections were made in the split mode (1:5).

The TMSO derivatives were formed following the method of Ruiz-Matute et al.[33] First, a volume of 450 μL of the resulting intestinal digesta, corresponding to 225.0 μg of saccharides was added to 200 μL of internal standard solution, containing 0.5 mg/mL of phenyl-β-glucoside. Afterward, the mixture was dried at 40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). Sugar oximes were formed by adding 250 μL hydroxylamine chloride (2.5%) in pyridine and heating the mixture at 70 °C for 30 min. Subsequently, the oximes obtained in this step were silylated with hexamethyldisylazane (250 μL) and trifluoroacetic acid (25 μL) at 50 °C for 30 min.[34] Derivatization mixtures were centrifuged at 6,700 x *g* for 2 min and supernatants were injected in the GC.

Data acquisition and integration were done using Agilent ChemStations software (Wilmington, DE, USA). Response factors were calculated after the duplicate analysis of standard solutions (fructose, galactose, glucose, lactose, lactulose, sucrose, raffinose and stachyose), at different concentrations ranging from 0.005 to 4 mg/mL.

Statistics. All digestions were carried out in duplicate and two GC-FID analysis were performed for each digestion treatment ($n = 4$). The comparisons of means using analysis of variance (ANOVA) were made using the statistical package (SPSS Inc., Chicago, Il). The differences were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

Determination of the main enzymatic activities of the RSIE. Table 1 shows the protein content and β-galactosidase, maltase, sucrase, trehalase, and palatinase activities of RSIE measured under the assayed digestion conditions. Maltase activity was the highest with 17-, 19-, 65-, and 134-fold increases as compared to β-galactosidase, sucrose, trehalase, and palatinase activities, respectively. These data are in agreement with the huge difference previously reported between the activities of maltase and the rest of the disaccharidases of the whole region of the small intestine of rats.[18] As a consequence, RSIE exhibited much more moderate β-galactosidase and sucrase activities with similar values of 26.7 U and 23.5 U, respectively. In contrast to these values, Oku et al.[18] observed a 4-fold increase of sucrase activity as compared to β-galactosidase activity in the small intestine. This dissimilarity could be attributed to several methodological factors, such as different assay and detection methods, and/or different substrates (lactose vs *o*-NPG) used in both studies. In addition, β-galactosidase activity gradually decreases during aging of the rat,[35] which could impair the comparison between different studies. Finally, RSIE showed low trehalase and palatinose activities whose specific values were in agreement with previous work.[18]

Table 1. Protein Content and Enzymatic Activities of Rat Small Intestine Extract (RSIE) Measured at the Studied Conditions[a]

Activity	Substrate	Conditions (pH; T)	U (μmol/min g)
β-galactosidase	*o*-NPG[b]	7.0; 37 °C	26.7 ± 2.0[c]
Maltase	*p*-NPG[b]	6.8; 37 °C	443.2 ± 2.0[d]
Sucrase	Sucrose[e]	6.8; 37 °C	23.5 ± 0.7[d]
Palatinase	Palatinose[e]	6.8; 37 °C	3.3 ± 0.4[d]
Trehalase	Trehalose[e]	6.8; 37 °C	6.8 ± 1.0[d]

[a]Protein content of RSIE: 8.9 ± 0.4% (w/w). [b]Enzyme activity determined by measuring the absorbance of released NP at 420 nm. [c]Values are expressed as means ± SD ($n = 6$). [d]Values are expressed as means ± SD ($n = 3$). [e]Enzyme activity determined by measuring the absorbance of released reducing sugars at 540 nm.

Small intestinal digestion of digestible carbohydrates. Figure 1 shows the evolution of maltose, sucrose, and lactose throughout the intestinal digestion process with the extract. Although substantial hydrolysis rates were observed in all cases, dissimilar trends were observed for each carbohydrate. Thus, maltose was rapidly and fully digested, as it disappeared after 15 min of digestion, which was the first sampling time (Figure 1A). Sucrose showed a slower but also high digestion rate, achieving a relative hydrolysis rate of 88.1% at the end of the digestion (Figure 1B), whereas lactose was the less hydrolyzed substrate with a maximum degradation of 55.8% (Figure 1C). Among the main human and rat dissacharidases, β-galactosidase has been reported to have the lowest activity, which could explain the lower hydrolysis rate of lactose as compared to maltose and sucrose.[18]

Small intestinal digestion of prebiotic carbohydrates. The digestibility of a total of six carbohydrates classified as well-known (lactulose, GOS-1, GOS-2, and FOS) or potential prebiotics (lactosucrose and OsLu) was assessed using RSIE and, conveniently, monitored and quantified by GC-FID in order to draw insights from the partial breakdown, if any, of the tested prebiotics.

Single carbohydrates, such as lactosucrose and, specially, lactulose, showed a high resistance to the intestinal digestion, resulting in low hydrolysis degrees of 26.0% and 11.1% (Table 2), respectively, after 2 h of digestion.

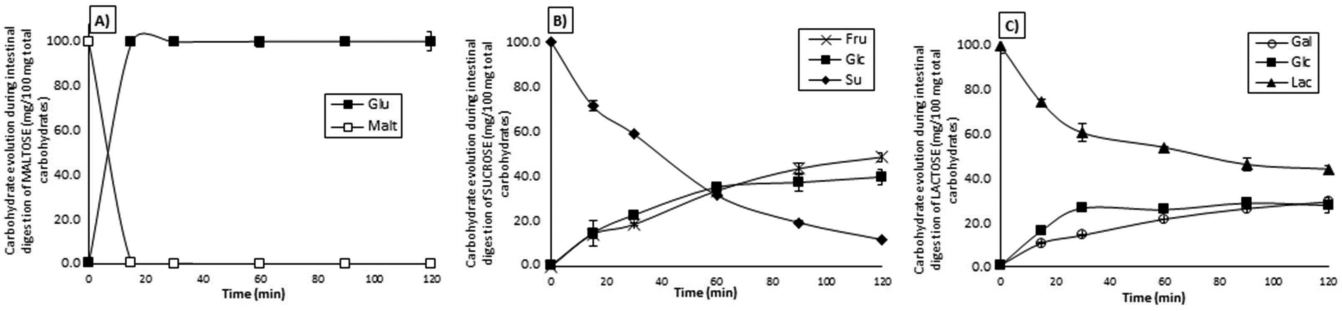

Figure 1. Hydrolysis rates of maltose (A), sucrose (B), and lactose (C) and their released monosaccharides upon small intestinal digestion at 37 °C, pH 6.8 for 2 h using RSIE.

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Table 2. Hydrolysis Degree[b] (%) of Nondigestible Carbohydrates during the Small Intestinal Digestion Treatment using RSIE at 37 °C, pH 6.8[a]

Digestion time (min)	Lactulose	GOS-1[c]	GOS-2[c]	OsLu[d]	FOS[e]	Lactosucrose
15	3.2 ± 0.5 a	13.2 ± 1.7 c	9.7 ± 1.5 b	2.5 ± 1.3 a	2.9 ± 0.1 a	7.4 ± 0.8 b
30	4.5 ± 0.3 a	21.1 ± 1.2 d	15.7 ± 1.5 c	6.2 ± 1.0 a	4.3 ± 0.2 a	10.4 ± 0.4 b
60	7.9 ± 0.1 a	30.1 ± 1.0 c	18.0 ± 1.1 b	8.1 ± 0.8 a	7.2 ± 0.3 a	18.0 ± 1.4 b
90	8.9 ± 0.2 a	34.2 ± 1.3 d	22.4 ± 1.7 c	16.0 ± 1.1 b	10.3 ± 0.4 a	21.4 ± 2.3 c
120	11.1 ± 0.1 a	34.2 ± 0.5 d	23.3 ± 0.7 c	18.0 ± 3.2 b	12.0 ± 0.6 a	26.0 ± 1.7 c

[a]Different letters indicate statistical differences between all tested carbohydrate samples at the same reaction time using a one-way analysis of variance (ANOVA) ($p < 0.05$) ($n = 4$). [b]Data are expressed as the mean ± SD ($n = 4$). [c]Hydrolysis degree (%) based on the joint digestibility of di-, tri-, and tetrasaccharide fractions. [d]Hydrolysis degree (%) based on the joint digestibility of di- and trisaccharide fractions. [e]Hydrolysis degree (%) based on the joint digestibility of kestose and nystose.

Remarkably, GOS-2, with $\beta(1 \rightarrow 6)$ the predominant linkage, showed a significantly higher overall resistance to intestinal digestion (23.3% of hydrolysis degree at the end of digestion) than GOS-1 (34.2% of hydrolysis degree), whose main linkage is $\beta(1 \rightarrow 4)$, highlighting the key role played by the glycosidic linkage involved in the oligosaccharide chain.

Novel galacto-oligosaccharides derived from lactulose (OsLu) presented an overall hydrolysis degree (i.e., 18%) which was significantly higher than that of lactulose only at longer digestion times (90 and 120 min), whereas no significant differences between both carbohydrates were observed during the first hour of digestion. Nevertheless, OsLu had a hydrolysis degree significantly lower than those of GOS-2 and, specially, GOS-1 throughout the digestion process (Table 2). OsLu and GOS-2 are mainly comprised of oligosaccharides containing $\beta(1 \rightarrow 6)$ as the main glycosidic linkage, but they differ in the presence of fructose at the reducing end of OsLu instead of glucose. Therefore, this result reveals that the monomer composition is also a critical factor for carbohydrate digestibility. These data are in good agreement with previous findings described by Hernández-Hernández et al.,[36] who reported a lower ileal digestibility of OsLu as compared to GOS following an *in vivo* approach using rats. In addition, also in line with our findings, these authors observed that $\beta(1 \rightarrow 6)$ and $\beta(1 \rightarrow 2)$ linkages between galactose and glucose monomers were significantly more resistant to *in vivo* gastrointestinal digestion than the $\beta(1 \rightarrow 4)$ linkage between galactose units within the GOS mixture. According to these comparative findings, it could be inferred that the *in vitro* digestion model developed in the present work is suitable for replacing *in vivo* rat models, stressing the usefulness of the RSIE as a reliable, simple, and cost-effective tool to assess carbohydrate digestibility. Recently, OsLu have also been shown to be more resistant to *in vitro* digestion than conventional GOS following their inclusion in milk.[21]

Finally, FOS, a mixture comprised of kestose and nystose, were also less prone to intestinal degradation than GOS-1 and GOS-2, showing a low hydrolysis degree of 12.0% after 2 h of digestion (Table 2). These data confirm the high resistance to mammalian digestive enzymes of $\beta(2 \rightarrow 1)$ linkages previously observed in FOS.[9,19] In addition, the overall hydrolysis degree obtained with the current *in vitro* digestion model is fairly similar to a previous *in vivo* study carried out with healthy humans and based on aspiration of the gut content at the terminal ileum.[37] Concretely, up to 89% of ingested FOS in a single meal was recovered in intact form. Consequently, these authors indicated that around 11% of FOS was hydrolyzed by either acidic conditions or digestive enzymes in the small intestine.

Table 3 shows the individual content in the monosaccharide fraction of all prebiotic oligosaccharides assayed, as well as the joint content of di-, tri-, and tetrasaccharide fractions in GOS and OsLu mixtures. In agreement with the overall hydrolysis degree displayed in Table 2, in GOS-1 the minor tetrasacharide fraction was substantially reduced and also, although at a lesser extent, the tri- and disaccharide fractions, whereas GOS-2 and OsLu presented substantial hydrolysis only in the disaccharide fraction (Table 3). Furthermore, Figures 2 and 3 illustrate the different resistance of the individual carbohydrates of GOS-1 and OsLu, respectively, to intestinal digestion based on their corresponding GC-FID profiles. Peaks 5 and 7, identified as 4′-galactosyl-lactose (β-D-Gal(1 → 4)-β-D-Gal-(1 → 4)-D-Glc) and 4′-digalactosyl-lactose (β-D-Gal(1 → 4)-β-D-Gal(1 → 4)-β-D-Gal-(1 → 4)-D-Glc), respectively,[32] were the main tri- and tetrasaccharide present in GOS-1 and clearly diminished after 2 h of RSIE digestion (Figure 2). However, peak 4, identified as allolactose (β-D-Gal-(1 → 6)-D-Glc), an isomer of lactose having a $\beta(1 \rightarrow 6)$ linkage, and the minor peak 8, identified as 6′-digalactosyl-lactose (β-D-Gal(1 → 6)-β-D-Gal(1 → 6)-β-D-Gal-(1 → 4)-D-Glc), appeared to be fully resistant to RSIE digestion. In contrast, no measurable differences were observed in any of the individual chromatographic peaks corresponding to the trisaccharide fractions of OsLu, and only very small decreases could be detected in the disaccharide fraction (Figure 3). Therefore, the contribution to the overall hydrolysis degree of OsLu displayed in Table 2 seems to be due basically to the disaccharide fraction (Table 3).

Regarding FOS, nystose was partially hydrolyzed and, probably, converted to kestose, as indicated by the slight increase found in fructose content and the nondetection of released glucose and/or the trisaccharide inulotriose (β-D-Fru(2→1)-β-D-Fru(2→1)-β-D-Fru) (Table 3). This could be indicative of a higher lability of the linkage $\beta(2 \rightarrow 1)$ when it is bonding fructose monomers instead of fructose and glucose in inulin-type FOS. Finally, the partial hydrolysis of lactosucrose gave rise to the release of similar levels of sucrose and lactose, indicating, thus, no particular preference of the digestive enzymes between the $\beta(1 \rightarrow 4)$ linkage of the lactose moiety and the $\beta(2 \rightarrow 1)$ linkage of the sucrose moiety.

To sum up, nine dietary carbohydrates, three digestible and six considered as nondigestible, were subjected to digestion using RSIE combined with physiological conditions (i.e., temperature and pH), and their hydrolysis products were comprehensively analyzed and quantified by GC-FID. The results confirmed the high and ready digestibility of maltose and sucrose, followed distantly by lactose. In any case, either the well-known or the potential prebiotics showed a higher resistance to RSIE digestion although partial hydrolysis at

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Table 3. Carbohydrate Content Determined by GC-FID Analysis in Nondigestible Oligosaccharides during the Small Intestinal Digestion Treatment Using RSIE at 37 °C, pH 6.8

		Carbohydrate content (mg/100 mg of total carbohydrates)[a]								
Sample	Reaction time	Fructose	Galactose	Glucose	Lactose	Lactulose	Disaccharides	Trisaccharides	Tetrasaccharides	Total OS[b]
Lactulose	blank	0.0 ± 0.0	0.0 ± 0.0	N.D.	N.D.	100.0 ± 0.0	N.D.	N.D.	N.D.	N.D.
	15 min	1.1 ± 0.1	2.1 ± 0.4	N.D.	N.D.	96.8 ± 0.5	N.D.	N.D.	N.D.	N.D.
	30 min	2.1 ± 0.2	2.4 ± 0.2	N.D.	N.D.	95.5 ± 0.3	N.D.	N.D.	N.D.	N.D.
	60 min	3.6 ± 0.1	4.3 ± 0.1	N.D.	N.D.	92.1 ± 0.1	N.D.	N.D.	N.D.	N.D.
	90 min	4.3 ± 0.1	4.4 ± 0.4	N.D.	N.D.	91.3 ± 0.5	N.D.	N.D.	N.D.	N.D.
	120 min	5.1 ± 0.1	6.1 ± 0.1	N.D.	N.D.	88.8 ± 0.1	N.D.	N.D.	N.D.	N.D.
GOS-1	blank	N.D.	1.2 ± 0.0	27.1 ± 0.5	16.5 ± 0.3	N.D.	24.3 ± 0.4	26.3 ± 0.4	4.6 ± 0.6	55.2 ± 0.7
	15 min	N.D.	7.9 ± 0.2	29.3 ± 1.0	14.8 ± 0.4	N.D.	20.7 ± 0.4	24.1 ± 0.3	3.1 ± 0.3	48.0 ± 1.0
	30 min	N.D.	12.5 ± 0.8	29.8 ± 0.7	14.1 ± 0.4	N.D.	20.0 ± 0.3	21.5 ± 0.5	2.1 ± 0.2	43.6 ± 0.9
	60 min	N.D.	16.6 ± 0.6	32.4 ± 1.0	12.4 ± 0.3	N.D.	17.2 ± 0.3	19.7 ± 0.3	1.7 ± 0.4	38.6 ± 0.9
	90 min	N.D.	22.2 ± 1.0	30.1 ± 1.4	11.3 ± 0.2	N.D.	17.1 ± 0.6	17.7 ± 0.2	1.4 ± 0.1	36.3 ± 0.8
	120 min	N.D.	23.7 ± 0.9	30.1 ± 0.8	10.6 ± 0.2	N.D.	16.7 ± 0.3	17.7 ± 0.4	1.7 ± 0.1	36.3 ± 0.3
GOS-2	blank	N.D.	9.2 ± 0.1	31.0 ± 0.1	26.0 ± 0.1	N.D.	13.5 ± 0.1	19.4 ± 0.1	0.9 ± 0.1	33.8 ± 0.1
	15 min	N.D.	14.0 ± 0.5	33.3 ± 0.6	22.1 ± 0.7	N.D.	11.5 ± 0.1	18.4 ± 0.6	0.6 ± 0.1	30.5 ± 0.6
	30 min	N.D.	16.3 ± 1.0	36.5 ± 1.5	18.7 ± 0.2	N.D.	10.6 ± 0.5	17.5 ± 1.6	0.4 ± 0.1	28.5 ± 1.2
	60 min	N.D.	18.4 ± 0.4	36.5 ± 1.3	17.3 ± 0.5	N.D.	9.4 ± 0.4	17.8 ± 0.3	0.6 ± 0.1	27.7 ± 0.5
	90 min	N.D.	18.1 ± 0.5	41.7 ± 0.1	13.9 ± 0.3	N.D.	7.9 ± 0.3	17.3 ± 0.4	1.0 ± 0.1	26.3 ± 0.6
	120 min	N.D.	20.2 ± 0.1	40.8 ± 1.8	12.9 ± 0.2	N.D.	7.9 ± 0.4	17.1 ± 1.2	0.8 ± 0.1	25.9 ± 1.5
OsLu	blank	0.6 ± 0.0	16.7 ± 0.3	N.D.	N.D.	34.7 ± 0.1	26.2 ± 0.1	21.3 ± 0.2	N.D.	47.5 ± 0.2
	15 min	0.9 ± 0.3	20.8 ± 0.9	N.D.	N.D.	31.9 ± 0.9	23.5 ± 0.8	22.8 ± 0.8	N.D.	46.5 ± 0.8
	30 min	0.9 ± 0.2	23.0 ± 0.8	N.D.	N.D.	31.4 ± 0.7	22.6 ± 0.6	22.0 ± 1.0	N.D.	44.6 ± 0.5
	60 min	1.2 ± 0.1	24.0 ± 0.7	N.D.	N.D.	31.1 ± 0.4	20.0 ± 0.3	23.7 ± 0.4	N.D.	43.7 ± 0.4
	90 min	2.2 ± 0.1	27.6 ± 0.8	N.D.	N.D.	30.2 ± 0.1	18.9 ± 0.5	21.1 ± 1.0	N.D.	39.9 ± 0.5
	120 min	2.0 ± 0.2	28.1 ± 1.4	N.D.	N.D.	30.6 ± 0.4	17.8 ± 0.1	21.0 ± 1.9	N.D.	38.9 ± 1.8
		Fructose	**Galactose**	**Glucose**	-	**Sucrose**	-	**Kestose**	**Nystose**	**Total FOS**
FOS	blank	0.3 ± 0.0	N.D.	N.D.		2.7 ± 0.1		65.2 ± 1.1	31.8 ± 1.2	97.0 ± 0.1
	15 min	2.5 ± 0.1	N.D.	N.D.		3.3 ± 0.2		65.1 ± 0.8	29.1 ± 0.8	94.2 ± 0.1
	30 min	3.8 ± 0.1	N.D.	N.D.		3.3 ± 0.2		65.4 ± 1.0	27.5 ± 1.2	92.8 ± 0.2
	60 min	6.6 ± 0.2	N.D.	N.D.		3.4 ± 0.1		66.6 ± 0.6	23.4 ± 0.5	90.0 ± 0.1
	90 min	9.8 ± 0.4	N.D.	N.D.		3.2 ± 0.2		67.0 ± 0.8	20.0 ± 0.9	87.0 ± 0.3
	120 min	11.6 ± 0.4	N.D.	N.D.		3.1 ± 0.2		67.4 ± 0.5	18.0 ± 0.1	85.4 ± 0.5
Lactosucrose		**Fructose**	**Galactose**	**Glucose**	**Lactose**	**Sucrose**	-	**Lactosucrose**	-	
	blank	0.1 ± 0.1	0.0 ± 0.0	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.0		98.8 ± 0.3		
	15 min	0.8 ± 0.1	0.6 ± 0.1	2.5 ± 0.4	1.6 ± 0.1	3.0 ± 0.1		91.5 ± 0.6		
	30 min	1.4 ± 0.1	1.5 ± 0.1	2.7 ± 0.4	2.1 ± 0.1	3.8 ± 0.1		88.5 ± 0.4		
	60 min	2.5 ± 0.2	2.9 ± 0.2	5.9 ± 0.9	3.3 ± 0.2	4.6 ± 0.1		81.0 ± 1.4		
	90 min	3.9 ± 0.1	4.2 ± 0.2	4.5 ± 2.3	4.6 ± 0.1	5.2 ± 0.1		77.7 ± 2.3		
	120 min	5.4 ± 0.3	5.5 ± 0.4	4.9 ± 0.9	5.9 ± 0.5	5.3 ± 0.2		73.1 ± 0.7		

[a]Data are expressed as the mean ± SD (n = 4). [b]Total oligosaccharide contents based on the sum of di-, tri-, and tetrasaccharide fractions.

J. Agric. Food Chem. 2017, 65, 8046−8053
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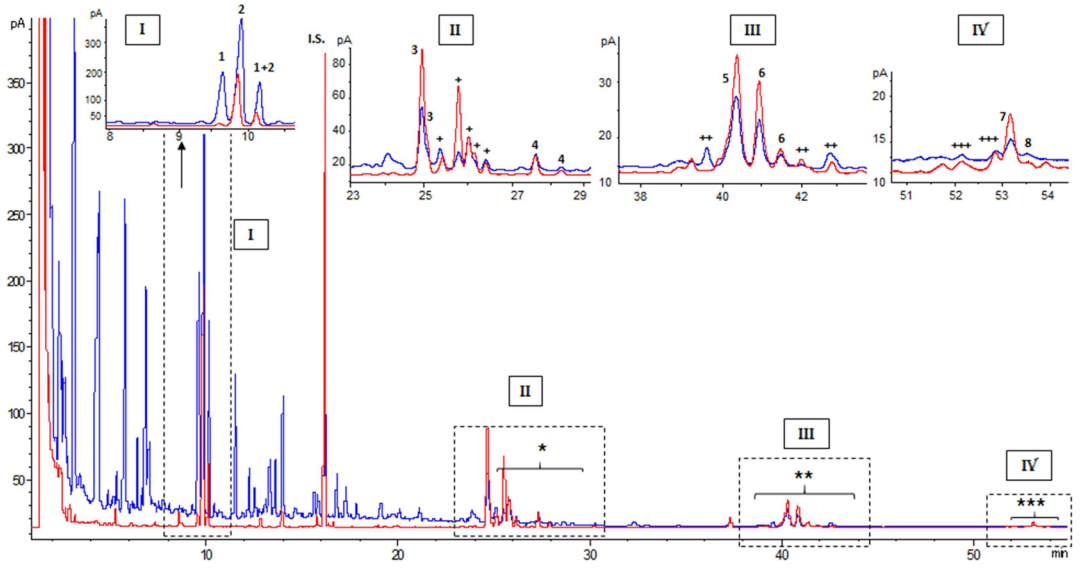

Figure 2. GC-FID profiles of TMSO derivatives of carbohydrates present in GOS-1 undigested (red) and after 2 h of small intestinal digestion with RSIE (blue). Peaks: 1: Galactose, 2: Glucose, I.S.: Internal standard (phenyl-β-D-glucoside), 3: Lactose, 4: Allolactose, 5: 4′-galactosyl-lactose, 6: 6′-galactosyl-lactose + unknown peak, 7: 4′-digalactosyl-lactose, 8: 6′-digalactosyl-lactose. +: other disaccharides, ++: other trisaccharides, +++: other tetrasaccharides. *: Disaccharides were considered the sum of peaks 4 and other disaccharides labelled as +. **: Trisaccharides were the sum of peaks 5, 6, and other trisaccharides. ***: Tetrasaccharides were quantified as the sum of peaks 7, 8, and other tetrasaccharides.

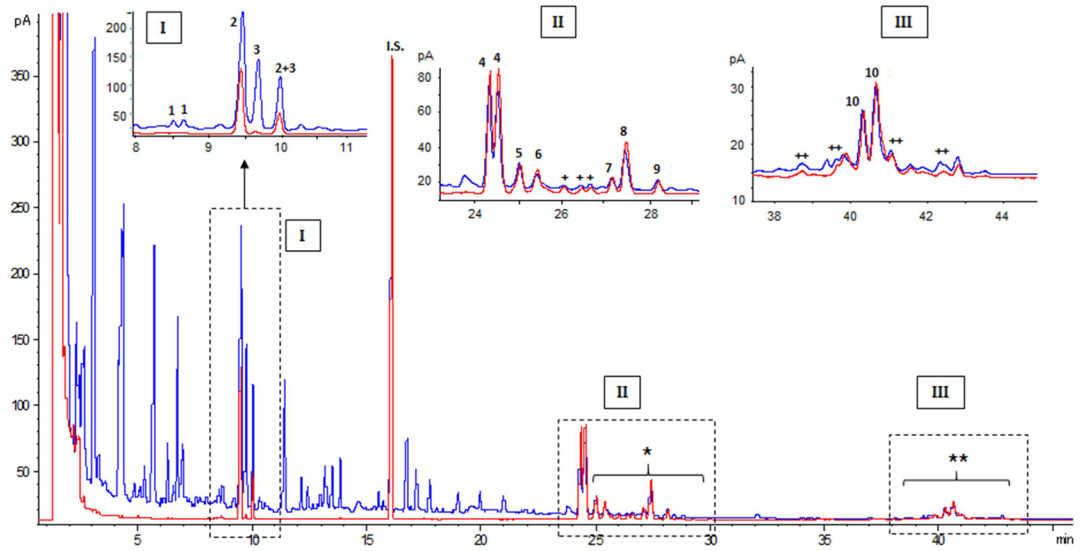

Figure 3. GC-FID profiles of TMSO derivatives of carbohydrates present in OsLu undigested (red) and after 2 h of small intestinal digestion with RSIE (blue). Peaks: 1: Fructose, 2: Galactose, 3: Glucose, I.S.: Internal standard (phenyl-β-D-glucoside), 4: Lactulose, 5: 1,1-galactobiose, 6: 1,3-galactobiose, 7: 1,1-galactosyl-fructose, 8: 1,6-galactobiose E, 9: 1,6-galactobiose Z, 10: 6′-galactosyl-lactulose. +: Other disaccharides, ++: Other trisaccharides. *Disaccharides were considered the sum of peaks 5 to 9. **Trisaccharides were the sum of peaks 10 and other trisaccharides.

different extents was observed in all tested carbohydrates. Thus, FOS (a mixture of kestose and nystose) and lactulose were the most resistant carbohydrates to intestinal digestion, followed closely by OsLu and, then, by GOS-2, lactosucrose, and, finally, GOS-1 (Tables 2 and 3). To the best of our knowledge, the present data are the first comparing the digestibility rates of two types of GOS differing in the predominant glycosidic linkage, revealing the higher resistance of $\beta(1\rightarrow6)$ compared to $\beta(1\rightarrow4)$ linkages to rat digestive enzymes. Moreover, the observed differences between OsLu and GOS-2 also pointed out the role of the monomer composition and, more concretely, the higher resistance of galactosyl-fructoses compared to galactosyl-glucoses.

There are currently very few studies and reliable data on the digestibility of potentially nondigestible carbohydrates, despite their increasingly important role in human health. The *in vitro* digestion model, based on the use of RSIE under physiological conditions of temperature and pH, described in this work has been shown to be a useful, simple, and cost-effective tool to evaluate the digestibility of dietary oligosaccharides. In general terms, the described method allows the distinction between digestible and nondigestible carbohydrates of degree of polymerization up to four, as the tested digestible carbohydrates were readily hydrolyzed whereas the oligosaccharides classified as nondigestible were barely or significantly less hydrolyzed than the digestible carbohydrates. The combination

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J. Agric. Food Chem. 2017, 65, 8046−8053

of RSIE digestion with sensitive and powerful separation methods, such as GC-FID, instead of colorimetric methods as has been traditionally performed, allows much more informative read-outs of the digestion process (e.g., lability of different glycosidic linkages, determination of the released carbohydrates resisting digestion). In addition, the developed *in vitro* digestion model has the advantage of requiring a minimum quantity of carbohydrates (0.5 mg), which is typically a limiting factor when the digestibility of novel carbohydrates produced at laboratory scale is assessed.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

AOAC, Association of Official Analytical Chemists; EFSA, European Food and Safety Authority; FOS, fructooligosaccharides; GOS, galactooligosaccharides; NDO, nondigestible oligosaccharides; OsLu, oligosaccharides derived from lactulose; RSIE, rat small intestine extract; TMSO, trimethylsilylated oximes

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Study on the digestion of milk with prebiotic carbohydrates in a simulated gastrointestinal model

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ABSTRACT

The behaviour of oligosaccharides from lactulose (OsLu) included with milk was examined during *in vitro* gastrointestinal digestion using the Infogest protocol as well as some small intestine rat extract. The digestion was compared with commercial prebiotics GOS and Duphalac®. Electrophoretic analysis demonstrated that the prebiotic carbohydrates did not modify the gastric digestion of dairy proteins. Similarly, no significant effect of gastrointestinal digestion was shown on the prebiotic studied. In contrast, under the intestinal conditions using a rat extract, the oligosaccharides present in OsLu samples were less digested (<15%) than in GOS (35%). Moreover, lactulose was more prone to digestion than their corresponding trisaccharides. These results demonstrate the limited digestion of OsLu and their availability to reach the large intestine as prebiotic.

1. Introduction

Prebiotics can reach the distal portions of the colon to selectively stimulate the growth of bifidobacteria and lactobacilli, providing important benefits to health (Gibson, Probert, Loo, Rastall, & Roberfroid, 2004). The most relevant compounds are oligosaccharides. These prebiotics may exert other bioactive properties such as improving mineral absorption and metabolic disorders and slow gastric emptying, among other effects (Moreno, Montilla, Villamiel, Corzo, & Olano, 2014).

Several commercial preparations of galactooligosaccharides (GOS) and fructooligosaccharides (FOS) are used as prebiotic ingredients in some foods such as infant formula and dairy products (Sabater, Prodanov, Olano, Corzo, & Montilla, 2016). Lactulose (i.e. lactose isomer) is also a recognized prebiotic for the treatment of constipation and systemic portal encephalopathy (Corzo-Martínez et al., 2013). Given the huge interest in recent years towards the gastrointestinal function and new structures with improved properties, new routes to obtain a second-generation of prebiotic oligosaccharides are being explored (Moreno, Corzo, Montilla, Villamiel, & Olano, 2017). This is the case of the oligosaccharides derived from lactulose (OsLu). These prebiotic mixtures, obtained by enzymatic synthesis using β-galactosidases from microbial origin, might impart better prebiotic properties than commercial GOS (Moreno et al., 2014).

One of the requirements for oligosaccharides to be considered as prebiotics is their resistance to digestion in the upper gastrointestinal tract. The susceptibility of prebiotic oligosaccharides to hydrolysis during their passage through the gastrointestinal tract is largely affected by the chemical structure and can impact their final state when they reach the colon to be fermented by the microbiota. Ohtsuka et al. (1990) found that the trisaccharide 4′-galactosyl-lactose was hardly digested *in vitro* with a homogenate of intestinal mucosa of rats. According to Torres, Gonçalves, Teixeira, and Rodrigues (2010), more than 90% of GOS are stable to digestive enzymes and can reach the colon to exert their positive effect. Carbohydrate analysis before and after exposure to certain protocols of *in vitro* digestion have shown that xylo-oligosaccharides, palatinose condensates, commercial GOS and lactulose were very resistant to hydrolysis, In contrast, lactosucrose, gentio-oligosaccharides, soybean oligosaccharides, fructo-oligosaccharide and inulin were slightly hydrolysed under such conditions (Playne & Crittenden, 2009).

To our knowledge, limited studies have been carried out on the digestibility of OsLu. Hernandez-Hernandez et al. (2012) pointed

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out in *in vivo* assays a higher resistance of OsLu compared to GOS during gastrointestinal digestion. This was ascribed to the presence of fructose in β(1 → 4) linkage with galactose at the reducing end of the OsLu molecules. However, there is a lack of studies on the susceptibility of OsLu to the gastrointestinal digestion when they are added in a food matrix and the impact of these compounds on the digestion of other food components. These considerations are important since standards would be more prone to changes as they are not protected in a food medium. Establishing the digestibility of prebiotic carbohydrates is of great practical application, since this influence the final dose of substrate that reaches the distal portions of gut to exert its prebiotic effect. Thus, the aim of this work has been to study the effect of the OsLu inclusion in milk on the digestion of proteins and the changes in the carbohydrate fraction using standardised *in vitro* digestive conditions with a more physiological relevant gastric digestion approach. A subsequent treatment with a rat small intestine extract has been included to study the effect of intestinal enzymes from mammals. The commercial prebiotics GOS and Duphalac® were also employed for comparison purposes.

2. Materials and methods

2.1. Chemicals and reagents

Galactose, glucose, fructose, lactose, lactulose, raffinose, stachyose, phenyl-β-glucoside and intestinal acetone powders from rat (rat intestine extract) from Sigma-Aldrich chemical Company (St Louis, MO).

2.2. Obtainment of prebiotic ingredients

OsLu were obtained at pilot scale by Innaves S.A. (Vigo, Spain) following the method described by Anadón et al. (2013). In brief, OsLu were synthesised using a commercial lactulose preparation (670 g/L; Duphalac®, Abbott Biologicals B.V., Olst, The Netherlands), diluted with water to 350 g/L and pH adjusted to 6.7 with KOH, and β-galactosidase from *Aspergillus oryzae* (16 U/mL; Sigma), selected by its high yield for synthesis of OsLu (Cardelle-Cobas et al., 2016). Enzymatic reactions were carried out at 50 °C in an orbital shaker at 300 rpm for 24 h. Afterwards, samples were immediately immersed in boiling water for 10 min to inactivate the enzyme. The mixture of oligosaccharides (20% [w/v]) was treated with fresh *Saccharomyces cerevisiae* (1.5% [w/v]; Levital, Paniberica de Levadura S.A., Valladolid, Spain) at 30 °C and aeration at 20 L/min, to decrease the monosaccharides content (Sanz et al., 2005). Finally, the samples were vacuum concentrated at 40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). GOS syrup was kindly provided by Friesland Campina Domo (Hanzeplein, The Netherlands).

2.3. Milk samples

Skim Milk Powder (low-heat organic, protein 42.34%, fat 0.89%, lactose 49.8% (w/w) (SMP) was kindly provided by Fonterra NZ. The SMP was reconstituted at 10% with distilled water and, subsequently, lactulose (Duphalac®), GOS or OsLu were added at 5% (w/w), taking into account previous recommendations for prebiotic doses (3.3 g of prebiotic carbohydrates/100 mL) (Lopez-Sanz, Montilla, Moreno, & Villamiel, 2015; Walton et al., 2012; Whisner et al., 2013). The samples were labelled as SMP + Duphalac®, SMP + GOS and SMP + OsLu and were kept refrigerated until subsequent assays.

2.4. In vitro gastrointestinal digestion

The solutions (see Fig. 1) used for the simulation of the oral and gastric phases were based on the standardised static digestion

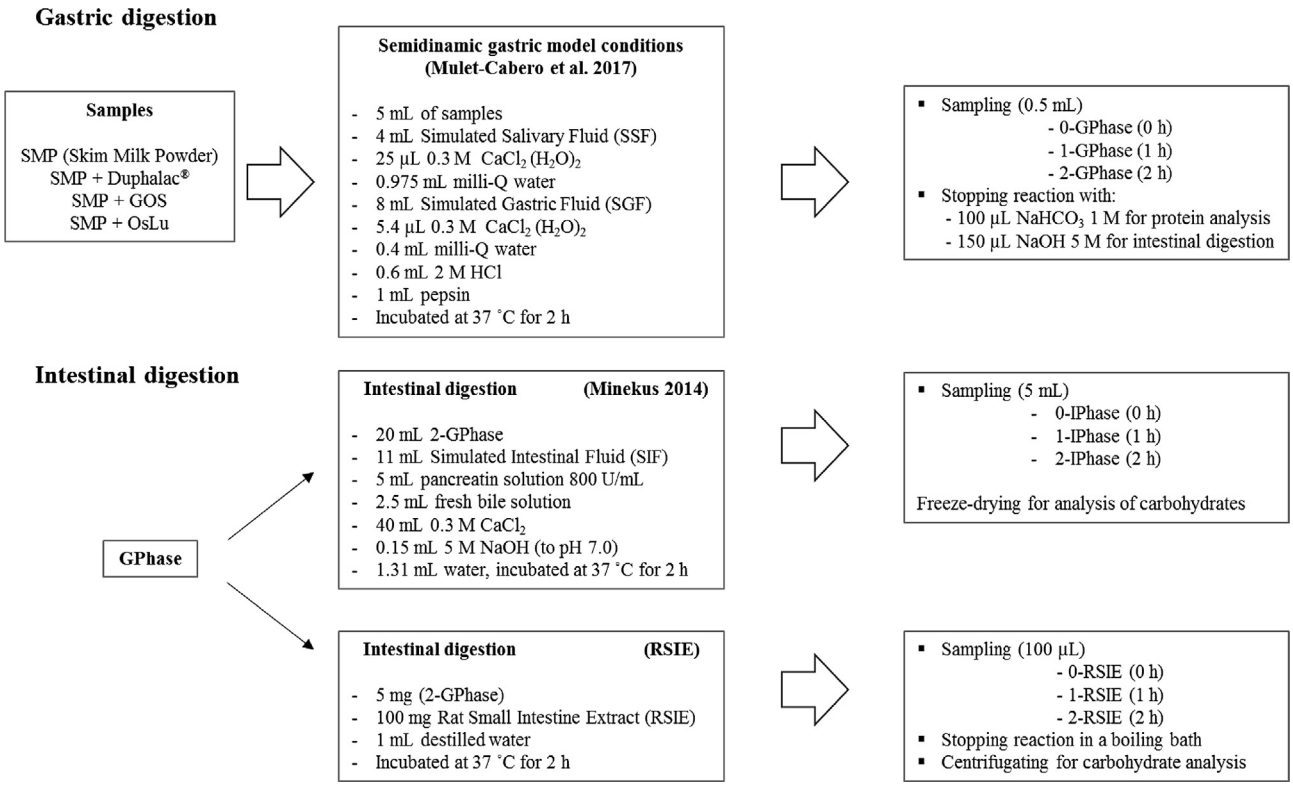

Fig. 1. Scheme of the experimental procedure.

protocol Infogest (Minekus et al., 2014). 5 mL of sample was placed into a 70 mL glass v-form vessel thermostated at 37 °C. To simulate the oral phase, 4 mL of Simulated Salivary Fluid (SSF, Table 1S, Verhoeckx et al., 2015), 25 μL 0.3 M $CaCl_2(H_2O)_2$ and 0.975 mL Milli-Q water were added and mixed for approximately 2 min using a 3D action shaker (Mini-gyro rocker-SSM3-Stuart, Barloworld Scientific limited, UK) at 35 rpm. The simulation of the gastric phase was conducted using a semi-dynamic model described by Mulet-Cabero, Rigby, Brodkorb, and Mackie (2017). The gastric fluids and enzyme solution were added gradually. Two solutions were added at a constant rate for 2 h: (1) 9 mL of a mixture consisted of 88.9% Simulated Gastric Fluid (SGF), 0.06% 0.3 M $CaCl_2(H_2O)_2$, 4.4% Milli-Q water and 6.7% 2 M HCl was added using the dosing device of an autotitrator (836 Titrando-Metrohm, Switzerland); and (2) 1 mL of pepsin (3214 U/mg solid, using haemoglobin as substrate) solution (in water) was added to reach the protease activity of 2000 U/mL in the final digestion mixture. This enzyme solution was added using a syringe pump (Harvard apparatus, PHD ultra, USA). The system was agitated using the 3D action shaker at 35 rpm during the digestion time.

The pH was recorded throughout the procedure. Samples (0.5 mL) were taken after 0, 1 and 2 h of digestion and the pepsin activity was stopped with 100 μL of 1 M $NaHCO_3$ for a subsequent analysis of the protein fraction and the rest of the sample with 150 μL of 5 M NaOH for the following intestinal digestion. This last sample was labelled as GPhase sample. After gastric digestion two different procedures for small intestinal digestion were carried out:

(i) 2 mL of GPhase was freeze-dried and kept at −20 °C until used for intestinal digestion assays with a crude enzyme of rat small intestine extract (RSIE). 5 mg of GPhase was mixed with 100 mg of RSIE and 1 mL distilled water. The mixture was incubated at 37° for 2 h, taking samples after 0, 0.5, 1 and 2 h. These samples were centrifuged at 10,000 rpm for 2 min and 100 μL of the supernatant was taken for carbohydrate analysis.
(ii) The rest of the liquid GPhase (~16.5 mL) was subjected to the small intestine conditions following the Infogest protocol (Minekus et al., 2014). The digestion was carried out at 37 °C for 2 h. Samples (5 mL) were taken at 0, 1 and 2 h of small intestinal digestion, which were respectively labelled as 0-IPhase, 1-IPhase and 2-IPhase. They were freeze-dried until further analysis.

2.5. Protein determination

The changes in the protein fraction during gastric digestion of milk containing prebiotic ingredients (GPhase 0, 1 and 2 h) were followed by SDS-PAGE. 65 μL of sample was mixed with 25 μL of 4X NuPAGE LSD sample buffer (Invitrogen, Carlsbad, California, USA) and 10 μL of 8% dithiothreitol. The mixture was heated at 70 °C for 10 min. 20 μL of mixture was loaded on a 12% polyacrylamide NuPAGE Novex Bis-Tris precast gel (Invitrogen, Carlsbad, California, USA) and RunBlue Precast SDS-PAGE gel cassette (Expedeon Ltd., Cambridgeshire, United Kingdom). SDS-PAGE was performed according to the manufacture's instructions. Mark 12 Unstained Standard (Invitrogen) was used as a molecular weight marker (ranging from 2.5 to 200 kDa).

2.6. Carbohydrate analysis by GC-FID

Trimethyl silylated oximes (TMSO) of carbohydrates (mono-, di- and oligosaccharides) present in samples were determined by Gas Chromatography following the method described by Montilla, Corzo, Olano, and Jimeno (2009). Samples corresponding to 0.5 mg of saccharides were added to 0.2 mL of Internal Standard (I.S.) solution which contained 0.5 mg/mL of phenyl-β-glucoside. Response factors respect to I.S. were calculated after the duplicate analysis of standard solutions (fructose, galactose, glucose, lactose, lactulose, sucrose, raffinose and stachyose), at different concentrations ranging from 0.005 to 4 mg/mL.

2.7. Statistical analysis

All digestions were carried out in duplicate and analyses were also performed in duplicate (n = 4). The comparison of means was carried out using one-way analysis of variance (Tukey HSD Multiple Range Test). Statistical analyses were performed using the SPSS statistical package (Inc., Chicago, Il). The differences were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Effect on protein digestion

Fig. 1S (complementary material) shows the pH profile of the different samples of SMP with the addition of prebiotic ingredients (Table 2S, carbohydrate composition analysed by GC-FID) during their digestion in the semi-dynamic gastric model. The initial pH values were close to 7 in all cases and gradually decreased to 1.8 at the end of the gastric digestion. In general, the profiles of the milk samples with prebiotic ingredients were similar to that of the SMP (no prebiotic ingredient added). The gradual lowering of pH enables the restructuring of the proteins due to acid induced coagulation to be simulated and is based on typical pH profiles measured *in vivo* (Malagelada, Go, & Summerskill, 1979).

The electrophoretic profile of proteins corresponding to samples 0, 1 and 2 h of gastric digestion are illustrated in Figs. 2 and 3. These figures show bands of pepsin, caseins, BSA, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). In the case of mixtures with OsLu and GOS at 0 h (Fig. 2) more intense bands appeared in the area corresponding to α-La, probably due to the formation of complexes between the protein and carbohydrates, which disappeared during the digestion. In general, after 2 h of

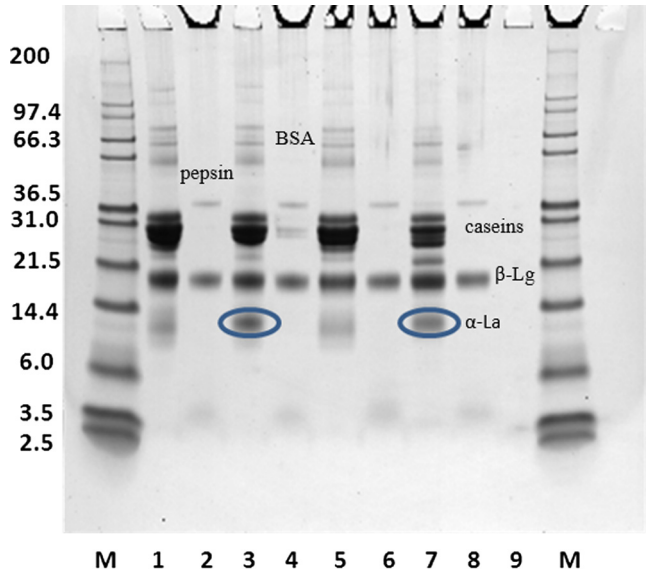

Fig. 2. Electrophoretic profiles of milk protein fractions (caseins, β-Lg, α-La, BSA) before and after 2 h of digestion (Bis-Tris-Gel, Novex, NuPage). M: Marker, 1: SMP 0 h, 2: SMP 2 h, 3: SMP + OsLu 0 h, 4: SMP + OsLu 2 h, 5: SMP + Duphalac® 0 h, 6: SMP + Duphalac® 2 h, 7: SMP + GOS 0 h, 8: SMP + GOS 2 h, 9: blank

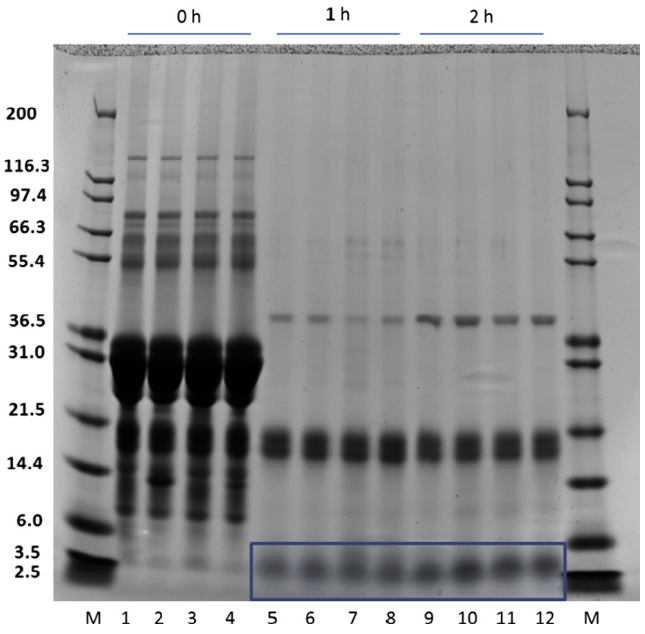

Fig. 3. Electrophoretic profiles of milk protein fractions (caseins, β-Lg, α-La, BSA) during 0, 1 and 2 h of digestion (RunBlue Precast gels). M: Marker; 1, 5 and 9 SMP; 2, 6 and 10 SMP + OsLu; 3, 7 and 11 SMP + GOS; 4, 8 and 12 SMP + Duphalac®. *Optical density was measured in the maximum of the peak with the Software Quantity One.

gastric digestion, the bands corresponding to undigested proteins from both SMP and SMP with added prebiotics were not detected with the exception of β-Lg which has been shown to be more resistant to pepsin hydrolysis (Mandalari et al., 2009). Fig. 3 shows some diffuse, low molecular weight bands in samples corresponding to 1 and 2 h of digestion which could be related to small molecular weight peptides formed after milk protein digestion (lanes 5–12). The intensity of these bands was estimated by the Quantity One software. This showed an increase of intensity with digestion time obtaining values of 0.54 at 0.62 after 1 h and 0.64 at 0.75 after 2 h, with the lowest values corresponding to skim milk control.

These results show that the SDS-PAGE profile of milk with prebiotic carbohydrates was similar to that of milk without addition of these ingredients, indicating that the presence of these prebiotics in milk at the concentration required to achieve a prebiotic effect, did not modify the gastric digestion of dairy proteins.

3.2. Effect on carbohydrate fraction

The effect of gastrointestinal digestion on the three different prebiotics, Duphalac®, GOS and OsLu included in milk was investigated. For this purpose, the samples from the semi-dynamic gastric model were subjected to two different intestinal digestion protocols, as indicated above (Infogest protocol or RSIE). In the case of the Infogest method, Fig. 2S (complementary material) illustrates, as an example, the chromatogram obtained by GC-FID of TMSO derivatives of carbohydrates present in the milk samples with OsLu after gastric digestion and the beginning of the intestinal phase (G + I 0 h). The peaks corresponding to carbohydrates with degree of polymerisation (DP) from 1 to 4 were found; among them galactose, lactulose and di-, tri- and tetrasaccharides derived from OsLu ingredient, and galactose, glucose and lactose from milk. Galactose was present in SMP with OsLu in higher proportion than in SMP with GOS (Table 1) in which the most abundant monosaccharide was glucose, due to their presence in the original prebiotic mixtures. In this respect, the addition of OsLu to milk or other products could be more interesting since OsLu presents lower proportion of caloric carbohydrates with lower glycaemic index than GOS (Lopez-Sanz et al., 2015). As observed in Table 1, SMP + Duphalac® had higher concentration of lactulose than SMP + OsLu because lactulose is used as substrate during its enzymatic hydrolysis and transgalactosylation.

Limited modifications were observed in the carbohydrate fraction following digestion using the Infogest protocol. In spite of the fact that there was a slight decrease of OS and trisaccharides in SMP + GOS after 2 h of digestion, these differences were not statistically significant. None of the carbohydrates derived from the prebiotic ingredients provided any significant change, indicating their stability during this enzymatic digestion by pancreatic fluids and bile salts. Moreover, it seems to be clear that the presence of other milk components did not impact the passage of GOS, Duphalac® and OsLu throughout the gastrointestinal digestion evaluated by the Infogest protocol.

In order to gain more insight in this subject and given that the Infogest protocol is mainly focus on the digestion of proteins, this study was completed with the evaluation of carbohydrate fraction of SMP with the three prebiotic ingredients after a subsequent digestion by means of an intestinal extract from rats, labelled as RSIE, as indicated in Materials and Methods section. Fig. 4A–D illustrates the evolution of each carbohydrate fraction in the SMP added with Duphalac®, GOS and OsLu after their gastric and intestinal (Infogest) and with RSIE (0.5, 1 and 2 h) of digestion. Data are expressed as % of hydrolysis, for lactose, lactulose and

Table 1
Carbohydrate evolution of milk samples during Intestinal digestion (G + I Phase), according to Infogest protocol.

		Carbohydrate content (%)							
		Galactose	Glucose	Lactulose	Lactose	Other Disaccharides	Trisaccharides	Tetrasaccharides	Oligosaccharides[a]
SMP	0 h	0.3 ± 0.1	0.4 ± 0.2	N.D.	99.4 ± 0.2	N.D.	N.D.	N.D.	N.D.
	1 h	0.3 ± 0.1	0.5 ± 0.1	N.D.	99.2 ± 0.1	N.D.	N.D.	N.D.	N.D.
	2 h	0.3 ± 0.0	0.4 ± 0.2	N.D.	99.4 ± 0.2	N.D.	N.D.	N.D.	N.D.
SMP + GOS	0 h	0.5 ± 0.1	7.6 ± 1.0	N.D.	65.6 ± 3.7	11.0 ± 0.8	12.9 ± 1.8	2.4 ± 0.6	26.4 ± 3.1
	1 h	0.5 ± 0.0	7.7 ± 1.5	N.D.	66.3 ± 3.3	12.0 ± 2.2	12.3 ± 1.4	3.3 ± 0.7	27.6 ± 4.2
	2 h	0.5 ± 0.0	6.9 ± 0.2	N.D.	68.4 ± 1.4	10.8 ± 1.3	10.9 ± 0.7	2.4 ± 1.7	24.1 ± 1.5
SMP + Duphalac®	0 h	3.6 ± 0.4	0.4 ± 0.4	22.0 ± 5,1	73.6 ± 4.9	N.D.	N.D.	N.D.	N.D.
	1 h	3.4 ± 0.8	0.2 ± 0.2	20.6 ± 1,1	76.5 ± 1.1	N.D.	N.D.	N.D.	N.D.
	2 h	3.1 ± 0.2	0.4 ± 0.2	21.6 ± 1,9	75.6 ± 1.7	N.D.	N.D.	N.D.	N.D.
SMP + OsLu	0 h	5.0 ± 0.3	0.3 ± 0.1	6.3 ± 2.1	68.4 ± 1.7	9.8 ± 0.3	9.3 ± 0.2	0.9 ± 0.2	20.1 ± 0.6
	1 h	5.0 ± 0.1	0.4 ± 0.2	7.1 ± 1.4	67.4 ± 1.3	9.8 ± 0.4	9.5 ± 0.4	0.8 ± 0.3	20.1 ± 0.3
	2 h	5.3 ± 0.3	0.3 ± 0.0	6.0 ± 0.4	69.0 ± 1.1	10.2 ± 0.5	8.6 ± 1.0	0.8 ± 0.6	19.6 ± 1.6

The data are expressed as the mean ± SD ($p > 0.05$). No statistical difference was determinates between 0, 1 and 2 h samples in all compounds using a one-way analysis of variance (ANOVA) (n = 4). N.D. No detected.

[a] Oligosaccharides: Values represent the sum of di-, tri- and tetrasaccharides.

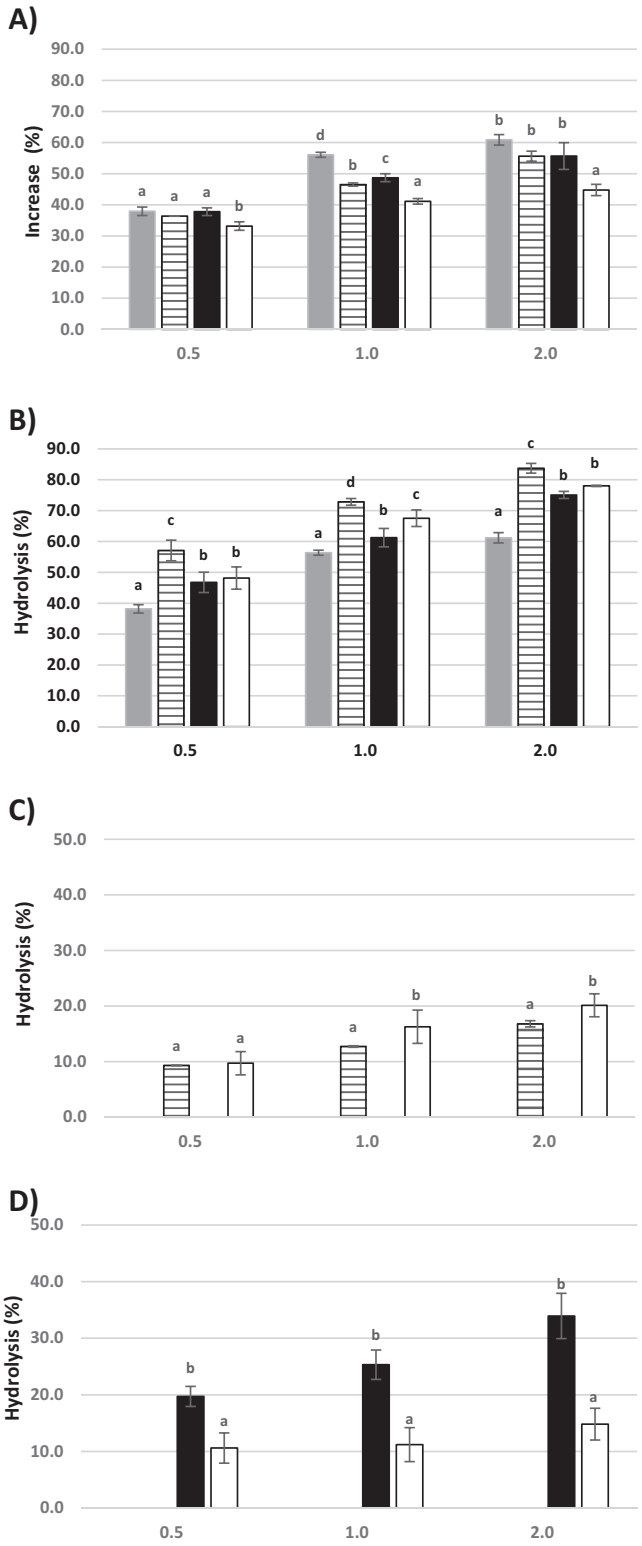

Fig. 4. Evolution of carbohydrates over time during the gastric and intestinal digestion with RSIE. Figure shows the results for each fraction analysed (A) Monosaccharides, (B) Lactose, (C) Lactulose and (D) Oligosaccharides after 0.5, 1.0 and 2.0 h of digestion. Grey bar represents SMP samples; Striped bar, SMP + Duphalac®; Black bar, SMP + GOS and White bar, SMP + OsLu. The results are shown as percentage of increase (A) or hydrolysis (B, C, D) relatively to their respective controls. Results are presented as mean ± SD (n = 4). Bar with different lower-case letters (a–d) represent statistical significant differences between each carbohydrate fraction at the same digestion time for their mean values at the 95.0% confidence.

oligosaccharides, and increase of monosaccharides, taking into account the control samples immediately taken after the addition of RSIE. The hydrolysis of compounds with DP ≥ 2 and mainly lactose increased with time of reaction, probably due to the presence of lactase (β-galactosidase) in the RSIE, in good agreement with the increase of the monosaccharide proportion.

In general, lactose was more hydrolysed than lactulose due to the presence of fructose instead of glucose in the β linkage of the latter (Olano & Corzo, 2009), being SMP + Duphalac® the sample with the highest degree of hydrolysis of lactose. In general, no significant differences ($p > 0.05$) were found for SMP samples with OsLu and GOS. Lactulose was significantly less susceptible to hydrolysis in SMP + Duphalac® than in SMP + OsLu. Furthermore, lactulose present in OsLu and Duphalac® was more prone to degradation than OS, probably ascribed to its lower Mw, although the difference was only significant after 1 h of digestion. Finally, OS were significantly more hydrolysed in SMP + GOS than in SMP + OsLu reaching values of 35% and 15%, respectively after 2 h; this was probably due to the more stable β(1–6) linkages in the OsLu mixture as compared to β(1–4) in GOS and the presence of fructose at the terminal end of molecule (Hernandez-Hernandez et al., 2012). These results indicate that OS (DP ≥ 3) present in OsLu were scarcely affected by the gastrointestinal digestion under the conditions used in the present work, being digested in a very low proportion in the small intestine which would favour the presence of a OS in the distal portions of colon to be fermented by beneficial bacteria.

To the best of our knowledge this is the first *in vitro* study on the digestion of prebiotics derived from lactose and lactulose as ingredients in a real food. The results obtained underline those of Hernandez-Hernandez et al. (2012) who pointed out, in *in vivo* assays with rats, that mixtures of OsLu were less digested than GOS. Particularly, the trisaccharide fraction of the former was 13% digested in the ileum, whereas in the latter case digestion was close to 53%. In both cases, the studied samples were the corresponding enzymatic mixtures obtained by transglycosylation and the presence of other food components was not considered. The small differences found in the total hydrolysis values with respect of our results could be ascribed to the differences in the experimental conditions.

4. Conclusions

According to the results obtained is possible to conclude that the presence of prebiotic carbohydrates in milk, at prebiotic doses, did not affect the gastric digestion of milk proteins, following the Infogest protocol. Similarly, under the same gastrointestinal digestion method, hardly any change was detected in the carbohydrate fraction of milk with GOS, Duphalac® and OsLu after 2 h of digestion. This might indicate the resistance of the three prebiotic mixtures, including OsLu, to gastric and pancreatic fluids and bile salts. However, when the digested samples of milk with prebiotics were subjected to intestinal digestion by a small gut intestinal extract of rat a dissimilar behaviour in the three cases was observed, OsLu samples being the most resistant to the action of enzymes present in the rat intestine extract, mainly in the case of OS fraction. These results highlight the possibility of OsLu to reach the large intestine, target organ, to exert their potential prebiotic effects.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.03.031.

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In vitro digestibility of galactooligosaccharides: Effect of the structural features on their intestinal degradation

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Abstract

Small intestinal brush border membrane vesicles (BBMV) from pig were used to digest galactooligosaccharides from lactose (GOS) and from lactulose (OsLu). Dissimilar hydrolysis rates were detected after digestion. Predominant glycosidic linkages and monomeric composition affected the resistance to intestinal digestive enzymes. β(1→3) GOS mixture was the most susceptible to hydrolysis (50.2%), followed by β(1→4) (34.9%), whereas β(1→6) linkages were highly resistant to digestion (27.1%). Monomeric composition provided a better resistance in β(1→6) OsLu (22.8%) as compared to β(1→6)-GOS (27.1%). This was also observed for β-galactosyl-fructoses and β-galactosyl-glucoses where the presence of fructose provided higher resistance to digestion. Thus, the resistance to small intestinal digestive enzymes highly depends on structure and composition of prebiotics. Increasing knowledge on this regard could contribute to the future synthesis of new mixtures of carbohydrates, highly resistant to digestion and with potential to be tailored prebiotic with specific properties, targeting, for instance, specific probiotic species.

Keywords: prebiotics, galactooligosaccharides, glycosidic linkages, *in vitro* digestion model, small intestine.

1. Introduction

Knowledge about the diversity of human microbiota and its relation to health has been largely gathered during last years. Moreover, there is a clear evidence suggesting that our microbiota is deeply implicated in a wide range of metabolic functions extending beyond the gut[1], such as, the regulation of the central nervous system homeostasis through immune, vagal and metabolic pathways [2,3,4] or the prevention of bone and respiratory diseases.[5,6] One of the most used strategies to modulate the composition and metabolic activity of microbiota is the use of prebiotics.[7]

Prebiotics definition refers to a "substrate that is selectively utilized by host microorganisms conferring a health benefit".[8] These compounds are characterized by the resistance to the digestion and acid conditions in the upper gastrointestinal tract and the ability to reach the colon without alteration in their structure.[9] To date, although a considerable number of compounds have been proposed as potential prebiotics, all well-recognized prebiotics are carbohydrates, mainly inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS) and lactulose. Among these, GOS have attracted growing interest due to the presence of galactose-based oligosaccharides, similar to those in human milk oligosaccharides (HMOs).[10]

GOS are commonly obtained by enzymatic synthesis from lactose by *β*-galactosidases and they are constituted by a complex mixture of galactoses linked by different linkages *β*(1→1), *β*(1→2), *β*(1→3), *β*(1→4) and *β*(1→6) and can vary from 1 to 8 units and a terminal glucose.[11] Composition of the obtained GOS mixture is deeply affected by several factors such as, the enzyme source, lactose concentration, substrate composition and reaction conditions (temperature, time and pH).[12,11,13] Galactooligosaccharides derived from lactulose (OsLu) have been also proposed as

63 emerging prebiotic compounds since they might provide enhanced prebiotic properties
64 compared to conventional GOS by increasing short-chain fatty acids content and the
65 population of *Bifidobacterium* and *Lactobacillus* species.[14,11] OsLu are obtained
66 similarly to GOS using lactulose as substrate and they are constituted by galactose units,
67 linked by a variety of glycosidic linkages ($\beta(1\rightarrow6)$, $\beta(1\rightarrow1)$ and/or $\beta(1\rightarrow4)$) determined
68 by the enzyme source, and a terminal fructose.[15]

69 The susceptibility of oligosaccharides to small intestinal digestion highly depends
70 on their structure, compromising their digestion fate and absorption.[16] However, ever
71 since prebiotics were first defined, most of the investigations have been carried out
72 focusing on their effect on the gut microbiota composition and/or activity, and few
73 efforts have been made towards the study of the resistance of these compounds to
74 digestion in the small intestine.[17-22] Moreover, the standardized official methods to
75 determine the digestibility of carbohydrates present several limitations, such as those
76 related to the matrix composition of the sample should be limited in complexity, the
77 lack of simulation of realistic enzyme substrate ratios and removal of digested products;
78 but most importantly, they do not take into consideration the disaccharidases that are
79 present in the small intestinal brush border membrane vesicles in mammals.[23-25]
80 Recently, the use of mammalian intestinal enzymes has been reported as an excellent
81 alternative method to determine carbohydrate digestion.[18,22,26]

82 *In vivo* and *in vitro* studies have described considerable digestion rates in the small
83 intestine of different types of GOS in rats (15-53% hydrolysis degree after 2 h of
84 digestion),[21,22,14,27,28] questioning the general acceptance that these compounds reach
85 intact the colon. These authors also have reported a different resistance to the upper
86 gastrointestinal tract conditions as well as a different effect on microbiota depending on
87 the main β-linkage in the mixture. Thus, $\beta(1\rightarrow6)$ linkages have been reported to be less

prone to degradation by intestinal enzymes and to exert better prebiotic effect as compared to other *β*-linkages.

Bearing that in mind, the aim of the present study was to evaluate the digestibility of recognized prebiotics such as GOS, with predominant *β*(1→3), *β*(1→4) or *β*(1→6) linkages, as well as emerging prebiotic candidates derived from lactulose (OsLu, *β*(1→6)) using small intestinal brush border membrane vesicles from pig.

2. Materials and methods

2.1 Chemical and reagents

D-Galactose (Gal), D-glucose (Glc), sucrose (*β*-D-Fru(2→1)-α-D-Glc), trehalose (α-D-Glc(1→1)-α-D-Glc), lactulose (*β*-D-Gal(1→4)-D-Fru), phenyl-*β*-glucoside, *o*-nitrophenyl (*o*-NP), p-nitrophenyl (*p*-NP), *o*-nitrophenyl-*β*-D-galactopyranoside (*o*-NPG) and *p*-nitrophenyl-α-glucopyranoside (*p*-NPG) standards were obtained from Sigma-Aldrich (St Louis, MO). Lactose (*β*-D-Gal(1→4)-D-Glc) was obtained from ACROS organics (Geel, Belgium) and fructose was obtained from Fluka analytical (St. Gallen, Switzerland). All standard carbohydrates were of analytical grade (purity ≥ 95%).

Kluyveromyces marxianus cells were kindly provided by Professor Robert Rastall from The University of Reading (United Kingdom). Nutritive medium (peptone, lactose and yeast extract) were supplied by Sigma-Aldrich.

2.2 Small intestinal brush border membrane vesicles (BBMV) preparation

Small intestinal brush border vesicles from six post-weaned pigs (7-10 months old) were obtained following methodology previously reported.[18,29] Briefly, three pig small

intestines, from the duodenum to the ileum, were obtained from a local slaughterhouse (Coca, Segovia, Spain). Immediately after sacrifice, the samples were kept at 4 ºC and transferred to the laboratory in less than 2 h. The small intestines were rinsed with cold phosphate buffered saline solution (PBS) (pH 7.3 – Oxoid; Basingstoke, UK), then slit open and scrapped with a glass slide. The mucose scrapped was suspended (1:1, w/v) in 50 mM mannitol dissolved in PBS at 4 ºC, homogenized during 10 min using a Ultra-Turrax® (IKA T18 Basic), adjusted with $CaCl_2$ to a final concentration of 10 mM and centrifuged at 3,000 *g* during 30 min. The supernatant was centrifuged at 27,000 *g* during 40 min and the resulting pellet, containing the BBMV, was re-suspended in buffer maleate (50 mM) pH 6.0 containing $CaCl_2$ (2 mM) and sodium azide (0.02%). Samples were lyophilized and kept at -80ºC.

2.3 Prebiotic oligosaccharides

OsLu were obtained at pilot plant scale by Innaves S.A. (Vigo, Spain) following the method described by López-Sanz et al. (2015).[30] Briefly, OsLu were synthesized using a commercial lactulose preparation (670 g/L; Duphalac, Abbott Biologicals B.V., Olst, The Netherlands), and a commercial preparation including *β*-galactosidase from *Aspergillus oryzae* (16 U/mL; Sigma) at pH 6.5, 50 ºC and 350 rpm during 24 h. In addition, three different commercially available GOS mixtures with predominant *β*(1→3) linkages GOS (named GOS-1), predominant *β*(1→4) linkages GOS (named GOS-2) and predominant *β*(1→6) GOS (named GOS-3), were tested.

2.3.1 Prebiotic oligosaccharides purification

Purification of prebiotic compounds was carried out by yeast treatment with *K. marxianus*.

K. marxianus cells were grown in YPD (1 % (w/v) yeast extract, 2 % peptone and 2 % lactose) (500 mL) at 37 ºC during 48 h. Samples were then centrifuged at 4,000 *g* for 10 min and washed three times on PBS (500 mL), supernatant was discarded, and washed samples were taken to incubation. Twenty-five mL of prebiotic ingredients (10% in PBS) and *K. marxianus* yeast (equivalent to 25 mL YPD) were incubated at 37 ºC for 48 h. Samples were then centrifuged at 4,000 x*g* for 20 min, filtered by 0.2 µm and then lyophilized and kept at -20ºC until analysis. Purification process was carried out three times for each sample (n=3) and monitored by GC-FID as explained below. GOS-1 mixture which was previously constituted by 30% monosaccharides, 22% lactose, 25% disaccharides and 23% trisaccharides (w:w) showed a loss of 67 % monosaccharides after *K. marxianus* treatment. GOS-2 was composed by 22% monosaccharides, 19% lactose, 8% disaccharides, 44% trisaccharides and 7% tetrasaccharides (w:w) showing a 97.4 % decrease in monosaccharides composition. GOS-3 composition which was 38% monosaccharides, 14% lactose and 52% oligosaccharides (w:w) showed a decrease of 95 % of monosaccharides. OsLu was constituted by 7.8% monosaccharides, 49.3% lactulose 28.8% disaccharides and 14.1% trisaccharides (w:w).

2.4 Small Intestinal BBMV characterization

Pig small intestinal BBMV (10 mg/mL) was homogenized in ice-cold 0.05 M sodium phosphate buffer solution and then centrifuged at 6,000 x*g* for 15 min. Supernatant was used as enzyme solution for determining protein content and enzymatic activity.

156 *2.4.1 Protein content determination*

157 Total protein content of the pig small intestinal BBMV was quantified according
158 to the Bradford method[31], using the Bio-Rad Protein Assay kit and bovine serum
159 albumin as a standard. The absorbance was monitored at 595 nm.

160 *2.4.2 Hydrolytic activities*

161 *2.4.2.1 β-galactosidase and maltase activities*

162 The determination of the pig intestinal *β*-galactosidase activity was adapted from
163 Warmerdam et al. (2014).[32] Briefly, a solution of *o*-NPG (0.5 mg/mL) in phosphate
164 buffer 0.05 M, pH 7.0 was prepared. The enzymatic activity was determined by
165 incubating 1,900 µL of the *o*-NPG solution and 100 µL of enzyme solution from BBMV
166 for 2 h at 37 °C. The method is based on the measurement of the continuous release of
167 *o*-NP from *o*-NPG. The absorbance of released *o*-NP was measured at 420 nm every 30
168 s using a spectrophotometer (Specord Plus, Analytik Jena) together with a temperature
169 controller (Jumo dTRON 308, Jumo Instrument Co.). The specific enzymatic activity
170 (U) was expressed in µmol min^{-1} g^{-1}, where one unit was defined as the amount of
171 enzyme that produced 1 µmol of *o*-NP in one min of reaction (biological replicates - n =
172 3). Similar procedure was used to determine the maltase activity by using a solution of
173 *p*-NPG in phosphate buffer 0.05 M, pH 6.8 (0.05% w/w) and monitoring the release of
174 *p*-NP at 420 nm every 20 s (n = 3).

175 *2.4.2.2 Sucrase and trehalase activities*

176 Sucrase and trehalase activities were determined following a method described
177 in a previous work.[21] A solution of sucrose or trehalose (0.5% w/v) in sodium phosphate
178 buffer 0.05 M, pH 6.5 was used. An eppendorf tube with 500 µL of sucrose or trehalose
179 solution was preheated at the reaction temperature, 37 °C. Subsequently, 200 µL of

enzyme solution was added and the mixture was incubated for 2 h and different aliquots were taken at different times (5, 10, 15, 30, 60, 90 and 120 min). Hydrolysis was stopped by adding 700 µL of a 3,5-dinitrosalicylic acid (DNS) solution. Sucrase and trehalase activity were determined measuring the reducing sugars released from the corresponding disaccharide hydrolysis at 540 nm, according to the DNS method.[33] The specific enzymatic activity (U) was expressed in µmol min^{-1} g^{-1}, where one unit was defined as the amount of enzyme that produced 1 µmol of reducing sugars in one min of reaction (n = 3).

2.5 In vitro digestion of prebiotic compounds with BBMV

The digestibility of three different types of GOS, OsLu and lactose and lactulose was evaluated using BBMV.

First, a solution of BBMV (10 mg/mL) in PBS solution, 6.8 pH, was prepared. Fifteen milliliters of this solution containing BBMVs (10 mg/mL) were placed in centrifuge tubes (two per sample) and prebiotic or disaccharides samples were added at a concentration of 0.2 mg/mL. Digestions were then initiated at 37 ºC during 5 h using 750 rpm in an orbital Thermomixer comfort (Eppendorf®). Aliquots of 1 mL (x2) were taken at 0, 1, 2, 3, 4 and 5 h of digestion and immediately heated in boiling water for 5 min to stop the reaction.

Furthermore, incubation of BBMV without any carbohydrate source was also analyzed. Results showed quantifiable amounts of glucose as the digestion proceeded. These values were conveniently substracted to avoid any overestimation of the monosaccharide fraction.

202 *2.6 Carbohydrates quantification by GC-FID*

203 Carbohydrates present in the samples and digested mixtures were analysed as
204 trimethylsilylated oximes (TMSO) by gas chromatography coupled to ionization flame
205 detector (GC-FID) following the method of Brobst & Lott Jr, (1966).[34] First, 500 μL of
206 samples (0.1 mg carbohydrates) was added to 500 μL of phenyl-*β*-glucoside (Internal
207 Standard, IS) and the mixture was dried in a rotary evaporator (Büchi Labortechnik AG,
208 Flawil, Switzerland). TMSO derivatives were formed by adding 250 μL of
209 hydroxylamine chloride in pyridine (2.5% w/v) and heating the mixture at 70 ºC for 30
210 min, followed by the addition of hexamethyldisilazane (250 μL) and trifluoroacetic acid
211 (25 μL) and incubated at 50 ºC for 30 min. Mixtures were centrifuged at 6,700 *g* for 2
212 min and supernatants were injected in the GC-FID.

213 TMSO derivatives were separated using a fused silica capillary column DB-5HT
214 (5%-phenyl-methylpolysiloxane; 30m x 0.25mm x 0.10μm, Agilent). Nitrogen at 1
215 mL/min was used as carrier gas. Injector and detector temperatures were set at 280 and
216 385 ºC, respectively. The oven temperature was set from 150 ºC to 380 ºC at a ratio of 3
217 ºC/min. Data acquisition and integration were done using Agilent ChemStation software
218 (Wilmington, DE, USA). Response factors were calculated after duplicate analysis of
219 standard solutions (fructose, glucose, galactose, lactose, lactulose and raffinose) over
220 the expected concentration range in samples, (0.005–1 mg) and IS (0.25 mg).

221 *2.7 Statistical Analysis*

222 Statistical analysis was carried out using SPSS for Windows, version 23.0. One-way
223 analysis of variance (ANOVA) and Tukey's *post hoc* test was used to determine
224 significant differences ($p < 0.05$) between concentrations of carbohydrates in each
225 prebiotic sample (n=3).

3. Results and Discussion

High decreases in monosaccharide composition was observed after *K. Marxianus* (Material and methods section 2.3.1). In this sense, monosaccharides are the major impurities in GOS obtainment, therefore, removal of these compounds is recommended mainly due to their undesirable caloric value and glycaemic index.[16] Furthermore, inhibition of *β*-galactosidase by glucose and galactose in transgalactosylation and hydrolysis reaction of carbohydrates was reported.[35]

3.1 BBMV enzymatic characterization

The brush border of the mammalian intestinal mucosa contains several key enzymes present as multienzyme complexes, i.e. sucrase-isomaltase, lactase-phlorizin hydrolase, maltase-glucoamylase and trehalase.[36] Accordingly, it is well reported the presence of those carbohydrases in the brush border of the intestinal mucosa of pig.[37,18,38] **Table 1** shows the protein content and main enzymatic activities (*β*-galactosidase, maltase, sucrase and trehalase) of BBMV measured under the assayed digestion conditions. Maltase activity (753.1 U/g) was the highest with ten-fold higher values than the other measured activities. This higher value can be ascribed to the multiple maltase activities carried out by different enzymatic complexes such as maltase-glucoamylase which has two catalytic sites able to hydrolyse maltose. This maltase activity is also present in both catalytic sites found in the complex sucrase-isomaltase. Both enzymatic complexes are the most abundant glycosidases in the small intestine. [39]

To date, some studies have characterized the carbohydrase activities of small intestinal enzymes in pigs[40,38,41,18], showing a clear predominance of maltase activity as compared to other activities, which agrees with the data obtained in this work.

3.2 Digestion of prebiotic carbohydrates by BBMV

Figure 1 shows GC-FID profiles of oligosaccharides before and after 5 h of digestion with BBMV. Differences were observed between the profiles of the three GOS mixtures, 1,4-galactobiose (*β*-Gal-(1→4)-Gal) and 1,6-galactobiose (*β*-Gal-(1→6)-Gal) were identified as peaks 2 and 5, respectively in all samples. *β*-Gal-(1→3)-Glc and allolactose (*β*-Gal-(1→6)-Glc), both isomers of lactose were also detected in all samples as peaks 3 and 4, respectively. Further structural differences were found in the trisaccharides fraction. *β*-1,4-Galactosyl-lactose (*β*-Gal-(1→4)-*β*-Gal-(1→4)-Glc, peak 6) was detected in all samples, *β*-1,6-galactosyl-lactose (*β*-Gal-(1→6)-*β*-Gal-(1→4)-Glc, peak 8) was found in GOS-2 and GOS-3 samples and *β*-1,3-galactosyl-lactose (*β*-Gal-(1→3)-*β*-Gal-(1→4)-Glc, peak 7) was only detected in GOS-1 mixture. Tetrasaccharides were also noticed in GOS-2 mixture (**Table 2)** and this fraction was mainly constituted by *β*-Gal-(1→4)-*β*-Gal-(1→4)-*β*-Gal-(1→4)-Glc and other tetrasaccharides not identified in this work.[42,11,43]

OsLu mixture was constituted by *β*(1→6) as the main glycosidic linkage and mostly by galactosyl galactoses (Gal-Gal) and galactosyl fructoses (Gal-Fru). *β*-(1→6)-galactosyl-lactulose (*β*-Gal-(1→6)-*β*-Gal-(1→4)-Fru) was identified as the main trisaccharide in the sample. In general, all assessed GOS and OsLu showed a diminution after BBMV digestion, although considerable differences among all studied samples were observed.

Tables 2 and 3 show the quantitative determination of individual carbohydrates in GOS and OsLu during digestion, respectively. A progressive increase in the level of monosaccharides was found in all samples as digestion proceeded, which was concomitant with the decrease in di- and trisaccharide fractions. Digestion of standard

273 solutions of lactose or lactulose with BBMV is also shown for comparative purposes.
274 As expected, lactose was much more prone to degradation than lactulose due to the
275 presence of fructose instead of glucose in the *β*-linkage of the latter.[44] Lactose
276 degradation in GOS samples was remarkably lower (50-68 %) when compared to the
277 standard solution (97 %) (**Table 1S, Supplementary Information)**, probably due to the
278 fact that the degradation of particular GOS trisaccharides or tetrasaccharides could
279 revert released lactose, as well as to the presence of other carbohydrates in the GOS
280 mixtures which might mitigate the straightforward digestion of lactose when is present
281 alone. Regarding lactulose digestion, the standard solution showed a slight lower
282 hydrolysis than that observed for lactulose present in OsLu (29.5 and 32.8 %,
283 respectively, after 5 h of digestion). Similar behaviour was obtained in a previous work
284 comparing the digestibility of prebiotics added to milk in an *in vitro* study with a small
285 intestine rat extract.[22]

286 Concerning disaccharides degradation, *β*-Gal-(1→3)-Glc and *β*-Gal-(1→6)-Glc
287 (allolactose) exhibited a slight decrease in their content after the BBMV digestion.
288 Allolactose (*β*(1→6)) was the most resistant to hydrolysis when compared to lactose
289 (*β*(1→4)) and *β*(1→3) structures. In this regard, it has been previously reported the high
290 resistance of allolactose to intestinal mucosa with less than 5% of hydrolysis compared
291 with lactose in an *in vitro* human assay[45] and in an *in vivo* study with rats.[14] Concerning
292 galactosyl galactoses, none of these carbohydrates provided any noticeable change,
293 indicating their stability during the digestion with BBMV. Indeed, an increase of these
294 compounds was found in some samples. Specifically, GOS-2 mixture showed an
295 increase of 4' and 6'-galactosyl galactose, respectively, suggesting the possible
296 breakdown of the *β*(1→4) linkage of the terminal glucose in their trisaccharide fraction.

297 Regarding OsLu disaccharides, high resistance of galactosyl galactoses was also
298 observed. Limited hydrolysis of galactosyl-fructoses was found, with β(1→6)-
299 galactosyl-fructose linkages as the lowest decrease among all determined disaccharides
300 (**Table 3**). According to Hernandez-Hernandez et al.[14] it is plausible that, in a similar
301 way to lactulose, the other galactosyl-fructoses can be highly resistant to digestion
302 within the mammalian small intestinal system. In line with our results, Julio-Gonzalez
303 et al. (2019)[46] have recently reported the potential higher resistance to mammalian
304 digestion of galactosyl-galactoses than galactosyl-glucoses.

305 Regarding trisaccharides fraction, data in **Table 2** shows that β(1→3)-galactosyl-
306 lactose in GOS-1 exhibited a higher hydrolysis than β(1→4)-galactosyl-lactose in GOS-
307 2 and β(1→6)-galactosyl-lactose in GOS-3. However, to provide more insight into the
308 effect on linkage on trisaccharides fraction, **Table 4** shows the hydrolysis degree of
309 each different linkage trisaccharide present in all samples. In addition, the slope of the
310 representation of hydrolysis degree (%) *vs* time (h), which could be considered as the
311 hydrolysis rate, is also shown. Taking into account a standard intestinal digestion time
312 of 2 h, the hydrolysis degree of trisaccharides showed β(1→3)-galactosyl-lactose
313 (hydrolysis rate of 21.9% as determined in GOS-1) to be more prone to degradation by
314 intestinal enzymes followed by β(1→4)-galactosyl-lactose (7.8-17.4%), whereas
315 β(1→6)-galactosyl-lactose (5.0-7.1%) and β(1→6)-galactosyl-lactulose (4.9%)
316 exhibited the highest resistance to hydrolysis.

317 Concerning oligosaccharides as a whole (that is, the sum of di, tri and
318 tetrasaccharides), the linkages β(1→6), abundant in GOS-3 and OsLu, demonstrated to
319 be the most resistant to intestinal degradation (**Figure 2, Table 1S, Supplementary**
320 **Information)**, where the presence of fructose at the reducing end of molecules provided

OsLu a slight better resistance to digestion with 22.8 % against 27.1 % of hydrolysis for GOS-3 after 5 h (**Figure 2C**). Furthermore, hydrolysis rate for GOS-3 and OsLu (**Table 5**) showed a lower degradation for OsLu as compared to GOS-3 after 2 and 5 h of digestion. GOS-2 oligosaccharides mixture was slightly more prone to degradation (34.9 %) with a higher hydrolysis rate after the BBMV digestion whereas GOS-1 oligosaccharides mixture exhibited the highest degree of hydrolysis with 50.1 % (**Table 1S)** degradation and the highest hydrolysis rate after 2 h (12.3) and 5 h (9.6) of treatment with BBMV from pig small intestine as compared to the other samples (**Table 5)**.

In this sense, a recent work highlighted the utility of a similar BBMV from pig small intestine to produce prebiotic GOS, and revealed that BBMV preferably synthesizes GOS linked by $\beta(1\rightarrow3)$ bonds, finding β-Gal-(1→3)-β-Gal-(1→4)-Glc as the main trisaccharide after comprehensive NMR analysis.[46] This study also pointed out no presence of β-Gal-(1→6)-β-Gal-(1→4)-Glc, whereas the β-Gal-(1→4)-β-Gal-(1→4)-Glc trisaccharide was present but only at trace amounts. These findings support the data obtained in the current work since the most abundant glycosidic linkages, formed when mammalian intestinal β-galactosidase act as transgalactosidase, are expected to be preferentially broken under hydrolytic conditions.

In the other hand, regarding monosaccharides release, galactose amounts were slightly higher compared to glucose release, probably due to the composition of the main oligosaccharides in the samples. **Table 2** showed that the highest hydrolysis of GOS-1 oligosaccharides produced a higher release of total monosaccharides (62 mg/100 mg of total carbohydrates) after 5 h of digestion as compared to GOS-2, GOS-3 and OsLu (34.6, 38.9 and 33.8 mg/100 mg total carbohydrates, respectively). In this sense,

345 the highest resistance of galactobioses and galactosyl-fructoses could affect positively to
346 regulate the caloric intake and diminish the possible absorption of free monosaccharides
347 in the small intestine, highlighting the key role of the monomer composition and type of
348 glycosidic linkage in prebiotic oligosaccharide samples.

349 Results obtained in this work have demonstrated that the use of small intestinal
350 BBMV from pig is a reliable and useful strategy to evaluate prebiotic carbohydrate
351 digestibility. Intestinal *in vitro* digestion with BBMV revealed the partial degradation of
352 recognized prebiotics such as lactulose, different mixtures of GOS and an emerging
353 prebiotic OsLu at considerably dissimilar levels. Our findings have revealed a stronger
354 resistance of $\beta(1\rightarrow6)$ linkages oligosaccharides to *in vitro* digestion when compared to
355 $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ linkages GOS. In general, $\beta(1\rightarrow3)$ followed by $\beta(1\rightarrow4)$ linkages
356 were more prone to small intestinal degradation using BBMV. This less resistance to
357 intestinal digestion was also found for galactosyl-glucose disaccharides as compared to
358 galactosyl-galactoses (galactobioses). The key role of monomer composition was also
359 underlined by the presence of fructose in OsLu mixture, providing, thus, a higher
360 resistance to digestion of galactosyl-fructoses. Findings described in this work could be
361 extrapolated to humans providing evidence on the structure-function relationship, as
362 well as an increase on the knowledge of the different resistance of β-linkages for the
363 sake of a future potential development of new tailored prebiotics. Moreover, the
364 observed hydrolysis with mammalian small intestinal enzymes of recognized prebiotics
365 could challenge the general belief that these compounds reach the colon without any
366 alterations in their structure. More investigation should be done in order to gain more
367 insight in the concept of prebiotics' digestibility.

368

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Abbreviations used

GOS, Galactooligosaccharides

OsLu, Oligosaccharides derived from lactulose

o-NPG, *o*-nitrophenyl-*β*-D-galactopyranoside

p-NPG, *p*-nitrophenyl-α-glucopyranoside

BBMV, Brush Border Membrane Vesicles

DP, Degree of Polymerization

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Table 1. Specific enzymatic activities and protein content of pig small intestinal BBMV.

Activity	Substrate	Conditions	U (μmol/min g)
β-galactosidase	*o*-NPG	7.0; 37 °C	70.1 ± 1.4
Maltase	*p*-NPG	6.8; 37 °C	753.1 ± 16.5
Sucrase	Sucrose	6.8; 37 °C	19.9 ± 2.2
Trehalase	Trahalose	6.8; 37 °C	21.4 ± 7.6

Protein content of BBMV was 7.3 ± 0.5 %
Data are expressed as means ± SD (n = 3)

Table 2. Carbohydrate content (mg/100 mg of total carbohydrates) determined by GC-FID analysis in GOS samples during the digestion with pig small intestinal brush border membrane vesicles at 37 °C, pH 6.8

Digestion time (h)	Galactose	Glucose	Lactose	β(1→4) Gb	β(1→3) Gal-Glc	β(1→6) Gb	Allolactose	Other Disaccharides	β(1→4) Gal-la	β(1→3) Gal-la	β(1→6) Gal-la	Other Trisaccharides	Σ DI	Σ TRI	Σ TETRA	OS[a]
Lactose																
0	0.7 ± 0.0	1.1 ± 0.0	98.2 ± 0.0	-	-	-	-	-	-	-	-	-	-	-	-	-
1	26.7 ± 4.5	17.3 ± 7.0	56.1 ± 7.2	-	-	-	-	-	-	-	-	-	-	-	-	-
2	37.1 ± 6.3	28.9 ± 0.7	34.0 ± 2.7	-	-	-	-	-	-	-	-	-	-	-	-	-
3	31.5 ± 7.8	38.4 ± 11.5	30.0 ± 11.0	-	-	-	-	-	-	-	-	-	-	-	-	-
4	39.8 ± 7.7	41.9 ± 2.4	18.3 ± 6.6	-	-	-	-	-	-	-	-	-	-	-	-	-
5	47.4 ± 4.5	49.8 ± 5.1	2.8 ± 0.4	-	-	-	-	-	-	-	-	-	-	-	-	-
GOS-1																
0	5.5 ± 0.0	5.5 ± 0.0	34.2 ± 0.0	1.3 ± 0.0	5.4 ± 0.0	2.5 ± 0.0	4.8 ± 0.0	8.3 ± 0.0	9.6 ± 0.0	15.5 ± 0.0*	-	7.4 ± 0.0	22.4 ± 0.0	32.4 ± 0.0	-	54.8 ± 0.0
1	13.1 ± 3.5	15.7 ± 3.7	24.9 ± 2.4	1.3 ± 0.5	4.5 ± 1.1	2.7 ± 0.8	4.8 ± 0.7	6.0 ± 3.1	7.8 ± 1.1	12.0 ± 3.1*	-	7.1 ± 0.6	19.3 ± 4.1	26.9 ± 2.0	-	46.2 ± 6.1
2	18.2 ± 3.0	16.6 ± 3.0	23.9 ± 1.9	1.3 ± 0.3	4.2 ± 0.3	2.1 ± 0.6	4.7 ± 0.6	6.1 ± 0.7	8.1 ± 3.0	8.7 ± 1.1*	-	6.2 ± 0.7	18.3 ± 0.8	23.0 ± 2.6	-	41.3 ± 1.8
3	23.6 ± 3.2	29.9 ± 7.1	20.8 ± 2.2	1.1 ± 0.4	3.5 ± 0.3	2.3 ± 0.5	4.1 ± 2.3	6.1 ± 2.4	4.4 ± 1.0	9.1 ± 4.6*	-	4.9 ± 1.6	17.3 ± 4.5	18.4 ± 7.2	-	35.7 ± 11.5
4	29.0 ± 1.2	21.5 ± 2.2	17.2 ± 1.2	1.2 ± 0.1	3.2 ± 0.3	2.1 ± 0.6	3.6 ± 0.6	4.9 ± 0.1	3.3 ± 0.1	8.2 ± 0.3*	-	5.9 ± 0.7	15.0 ± 0.6	17.4 ± 1.0	-	32.3 ± 1.2
5	33.1 ± 4.0	28.9 ± 0.6	10.7 ± 2.1	1.2 ± 0.2	2.8 ± 0.3	2.1 ± 0.3	3.3 ± 0.2	3.2 ± 0.5	3.1 ± 0.1	6.0 ± 0.5*	-	4.7 ± 0.1	12.6 ± 0.2	14.7 ± 0.4	-	27.3 ± 0.7
GOS-2																
0	0.6 ± 0.0	0.0 ± 0.0	1.8 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.0	20.4 ± 0.0	-	18.2 ± 0.0	38.5 ± 0.0	4.1 ± 0.0	77.1 ± 0.0	16.4 ± 0.0	97.6 ± 0.0
1	8.6 ± 1.2	6.9 ± 0.2	1.7 ± 0.1	2.4 ± 0.6	0.9 ± 0.5	0.4 ± 0.1	3.2 ± 0.5	2.9 ± 0.6	15.0 ± 0.9	-	16.2 ± 1.0	26.9 ± 1.1	9.9 ± 0.7	58.1 ± 3.2	14.9 ± 0.5	82.9 ± 1.2
2	13.5 ± 1.0	9.5 ± 1.1	1.6 ± 0.5	2.2 ± 0.2	1.1 ± 0.1	0.4 ± 0.2	5.0 ± 0.9	3.1 ± 1.1	13.3 ± 1.3	-	15.6 ± 0.9	25.3 ± 0.9	11.7 ± 1.1	54.1 ± 2.0	12.8 ± 1.1	78.6 ± 0.9
3	16.0 ± 0.7	10.9 ± 0.2	1.3 ± 0.4	2.1 ± 0.5	1.2 ± 0.2	0.3 ± 0.3	5.8 ± 1.0	3.0 ± 0.5	10.5 ± 0.7	-	14.3 ± 0.7	23.7 ± 1.3	12.5 ± 0.4	48.5 ± 1.5	10.8 ± 1.3	71.8 ± 1.3
4	18.3 ± 0.3	12.3 ± 0.5	1.2 ± 0.3	2.4 ± 1.1	1.2 ± 0.3	0.3 ± 0.1	6.8 ± 0.7	3.9 ± 0.4	9.7 ± 0.4	-	14.1 ± 0.5	18.4 ± 0.8	14.5 ± 1.2	42.1 ± 0.9	10.5 ± 0.9	67.2 ± 0.7
5	21.2 ± 1.5	13.4 ± 1.0	0.9 ± 0.1	2.5 ± 0.8	1.0 ± 0.4	0.4 ± 0.2	8.0 ± 0.8	3.3 ± 0.2	8.8 ± 1.1	-	13.5 ± 0.3	16.1 ± 0.7	15.1 ± 0.9	38.4 ± 1.4	10.1 ± 1.1	63.5 ± 1.6
GOS-3																
0	0.7 ± 0.0	1.1 ± 0.0	25.1 ± 0.0	1.6 ± 0.0	4.1 ± 0.0	2.6 ± 0.0	9.0 ± 0.0	9.0 ± 0.0	9.4 ± 0.0	-	32.1 ± 0.0	5.3 ± 0.0	26.4 ± 0.0	46.8 ± 0.0	-	73.1 ± 0.0
1	5.9 ± 1.6	4.5 ± 1.0	20.1 ± 1.5	1.1 ± 0.1	3.1 ± 0.1	2.9 ± 0.2	9.2 ± 1.0	6.6 ± 0.2	8.2 ± 1.3	-	31.0 ± 2.2	6.2 ± 1.0	22.9 ± 1.4	45.4 ± 2.6	-	68.3 ± 1.2
2	10.3 ± 0.9	8.5 ± 1.7	16.1 ± 1.5	1.0 ± 0.0	2.5 ± 0.1	3.1 ± 0.3	9.2 ± 0.3	6.4 ± 0.2	7.7 ± 1.5	-	28.9 ± 1.7	6.2 ± 0.7	22.2 ± 0.2	42.8 ± 2.3	-	65.1 ± 2.2
3	13.7 ± 0.4	11.0 ± 0.1	11.6 ± 0.6	0.9 ± 0.1	2.4 ± 0.4	3.0 ± 0.2	8.7 ± 1.1	6.2 ± 0.6	7.1 ± 1.5	-	29.2 ± 1.1	6.2 ± 0.7	21.2 ± 0.8	42.5 ± 0.6	-	63.7 ± 0.4
4	18.3 ± 1.2	15.6 ± 0.5	9.8 ± 0.4	0.9 ± 0.2	2.2 ± 0.3	2.9 ± 0.1	8.3 ± 2.0	5.5 ± 0.2	6.7 ± 0.2	-	25.1 ± 1.0	4.7 ± 0.6	19.8 ± 1.7	36.5 ± 1.3	-	56.3 ± 0.9
5	21.9 ± 0.3	17.0 ± 1.2	7.9 ± 0.1	0.9 ± 0.1	2.0 ± 0.5	3.4 ± 0.0	6.8 ± 0.2	4.6 ± 0.7	5.1 ± 0.5	-	26.7 ± 1.1	3.7 ± 0.0	17.8 ± 0.4	35.5 ± 1.2	-	53.3 ± 1.6

Data are expressed as the mean ± SD (*n* = 4).
Gb = galactobiose (β-Gal-(1→4/6)-Gal)
Gal-la = galactosyl-lactose (β-Gal-(1→4/6)-β-Gal-(1→4)-Glc)
[a] Oligosaccharides content based on the sum of di-, tri- and tetrasaccharides.
*Represents the peak constituted mainly by β-1,3 galactosyl-lactose and traces of β-1,6 galactosyl-lactose

Table 3. Carbohydrate content (mg/100 mg of total carbohydrates) determined by GC-FID analysis in OsLu samples during the digestion with pig small intestinal brush border membrane vesicles at 37 °C, pH 6.8

Digestion time (h)	Fructose	Galactose	Lactulose	β(1→4) Gb	β(1→3) β(1→2) Gb	β(1→6) Glc-Fru	β(1→1) Gal-Fru	β(1→6) Gb	Other Disaccharides	β(1→6) Gal-lu	Other Trisaccharides	Σ DI	Σ TRI	OS[a]
Lactulose														
0	5.1 ± 0.0	5.5 ± 0.0	89.5 ± 0.0	-	-	-	-	-	-	-	-	-	-	-
1	8.1 ± 3.2	8.0 ± 2.5	83.9 ± 4.2	-	-	-	-	-	-	-	-	-	-	-
2	10.4 ± 2.7	11.2 ± 2.2	78.4 ± 4.1	-	-	-	-	-	-	-	-	-	-	-
3	11.9 ± 1.0	14.2 ± 1.0	74.0 ± 2.4	-	-	-	-	-	-	-	-	-	-	-
4	15.2 ± 2.0	16.7 ± 1.3	68.1 ± 3.1	-	-	-	-	-	-	-	-	-	-	-
5	18.1 ± 0.4	18.9 ± 0.3	63.0 ± 0.5	-	-	-	-	-	-	-	-	-	-	-
OsLu														
0	1.2 ± 0.0	6.6 ± 0.0	49.3 ± 0.0	6.9 ± 0.0	5.7 ± 0.0	2.9 ± 0.0	5.5 ± 0.0	5.3 ± 0.0	2.5 ± 0.0	8.2 ± 0.0	5.9 ± 0.0	28.8 ± 0.0	14.1 ± 0.0	42.9 ± 0.0
1	2.3 ± 0.2	12.5 ± 2.1	44.0 ± 0.6	6.4 ± 0.3	5.4 ± 0.9	3.1 ± 0.0	5.6 ± 0.2	5.4 ± 0.2	2.2 ± 0.3	7.9 ± 0.8	5.4 ± 0.1	28.1 ± 1.1	13.2 ± 0.5	41.2 ± 1.5
2	3.4 ± 0.2	17.5 ± 0.3	40.4 ± 1.5	5.9 ± 0.3	5.1 ± 1.0	2.9 ± 0.8	5.2 ± 0.2	4.9 ± 0.1	2.0 ± 0.7	7.4 ± 1.1	5.1 ± 0.3	25.9 ± 1.0	12.5 ± 0.1	38.4 ± 1.0
3	4.7 ± 0.4	20.5 ± 1.1	37.5 ± 1.0	5.6 ± 0.6	4.6 ± 0.3	2.8 ± 0.1	5.1 ± 0.1	4.9 ± 0.1	2.0 ± 0.1	7.3 ± 0.2	5.0 ± 0.1	25.0 ± 0.6	12.3 ± 0.3	37.3 ± 0.8
4	5.0 ± 0.5	24.5 ± 0.5	35.7 ± 0.5	5.4 ± 0.2	4.5 ± 0.7	2.5 ± 0.5	4.7 ± 0.1	4.5 ± 0.2	1.7 ± 0.5	7.1 ± 0.5	4.4 ± 0.6	23.3 ± 0.5	11.4 ± 0.9	34.7 ± 0.9
5	7.1 ± 0.2	26.7 ± 2.4	33.1 ± 1.5	5.6 ± 0.6	4.3 ± 0.3	2.6 ± 0.1	4.6 ± 0.1	4.5 ± 0.1	1.1 ± 0.0	6.6 ± 0.8	4.0 ± 0.9	22.6 ± 0.5	10.5 ± 1.3	33.1 ± 1.4

Data are expressed as the mean ± SD (*n* = 4).
Gb = galactobiose (β-Gal-(1→1/2/3/4/6)-Gal)
Gal-lu = galactosyl-lactulose (β-Gal-(1→6)-β-Gal-(1→4)-Fru)
[a] Oligosaccharides content based on the sum of di- and trisaccharides.

Table 4. Hydrolysis degree (%) evolution of different linkage trisaccharides (Tri) in each sample during the *in vitro* digestion with pig small intestinal BBMV.

	GOS-1		GOS-2		GOS-3		OsLu
Digestion time (min)	β(1→3) Gal-la	β(1→4) Gal-la	β(1→4) Gal-la	β(1→6) Gal-la	β(1→4) Gal-la	β(1→6) Gal-la	β(1→6) Gal-lu
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	22.6	18.8	26.5	11.0	12.8	3.4	3.7
2	43.9	15.6	34.8	14.3	18.1	10.0	9.8
3	41.3	54.2	48.5	21.4	24.5	9.0	11.0
4	47.1	65.6	52.5	22.5	28.7	21.8	13.4
5	61.3	67.7	56.9	25.8	45.7	16.8	19.5
Hydrolysis rate (after 2 h)	**21.9**	**7.8**	**17.4**	**7.1**	**9.0**	**5.0**	**4.9**

Table 5. Hydrolysis rate of oligosaccharides (sum of di- and trisaccharides)* in samples during the *in vitro* digestion with pig small intestinal BBMV.

	GOS-1 OS	GOS-2 OS*	GOS-3 OS	OsLu OS
Hydrolysis rate (after 2 h)	**12.3**	**9.7**	**5.5**	**5.2**
Hydrolysis rate (after 5 h)	9.6	6.6	5.3	4.6

* In the case of GOS-2, tetrasaccharides were also considered.

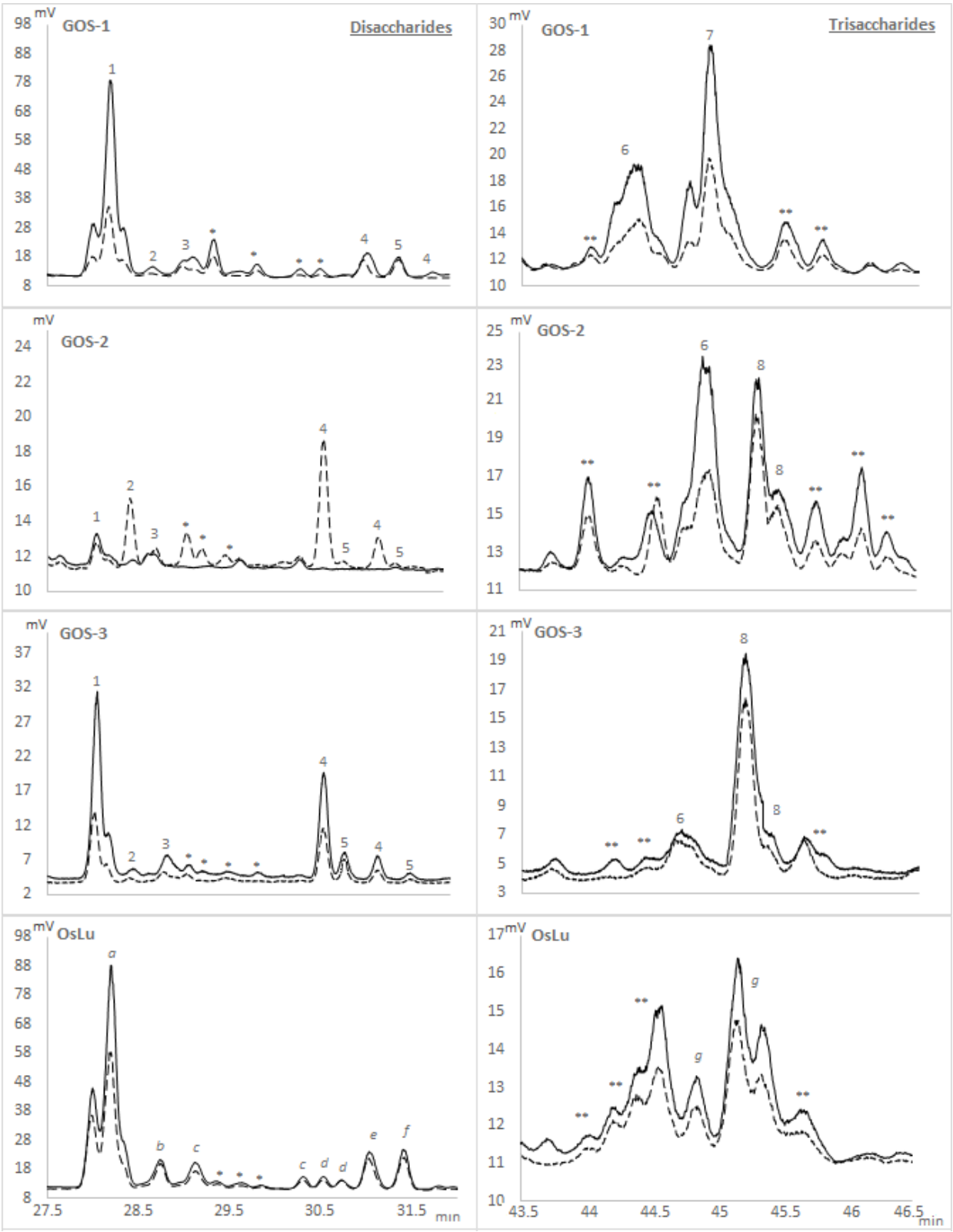

Figure 1. Chromatographic profiles of TMSO derivatives of prebiotics oligosaccharides before (continuous line) and after 5 h of digestion with pig small intestinal BBMV (striped line). GOS disaccharides: **1**, lactose; **2**, 1,4-galactobiose; **3**, β-Gal-(1→3)-Glc; **4**, allolactose; **5**, 1,6-galactobiose and ***** other disaccharides. GOS trisaccharides: **6**, β-Gal-(1→4)-β-Gal-(1→4)-Glc; **7**, β-Gal-(1→3)-β-Gal-(1→4)-Glc; **8**, β-Gal-(1→6)-β-Gal-(1→4)-Glc and ****** other trisaccharides. OsLu disaccharides: ***a***, lactulose; ***b***, 1,4-galactobiose; ***c***, 1,2-galactobiose+1,3-galactobiose; ***d***, β-Glc-(1→6)-Fru; ***e***, β-Glc-(1→1)-Fru; ***f***, 1,6-galactobiose and ***** other disaccharides. OsLu trisaccharides: ***g***, β-Gal-(1→6)-β-Gal-(1→4)-Fru and ****** other trisaccharides.

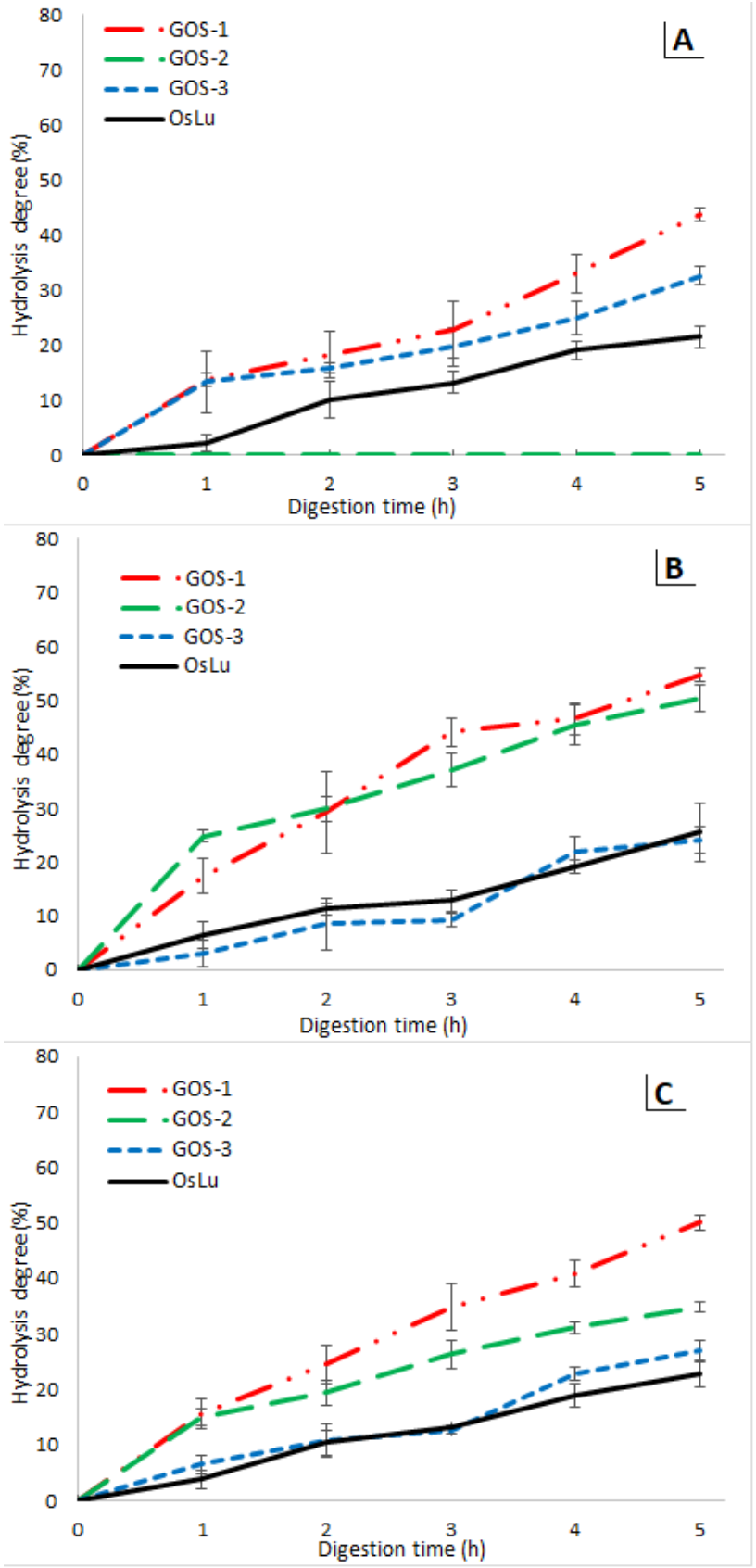

Figure 2. Evolution of hydrolysis (%) of carbohydrates during digestions with pig small intestinal BBMV from pig at 37 ºC, pH 6.8. Disaccharides (A), trisaccharides (B) and oligosaccharides (C). The later were expressed as the sum of di- and trisaccharides in GOS-1 and GOS-3; tetrasaccharides were also included in GOS-2.

Table 1S. Hydrolysis degree (%) of di-, tri, oligosaccharides and lactose, lactulose in all samples during digestion with pig small intestinal BBMV.

Σ Disaccharides					
Digestion time (min)		**GOS-1**	**GOS-2**	**GOS-3**	**OsLu**
	0	0	0	0	0
	1	13.8	0.0	13.3	2.4
	2	18.3	0.0	15.9	10.1
	3	22.8	0.0	19.7	13.2
	4	33.0	0.0	25.0	19.1
	5	43.7	0.0	32.6	21.5
Σ Trisaccharides					
Digestion time (min)		**GOS-1**	**GOS-2**	**GOS-3**	**OsLu**
	0	0	0	0	0
	1	17.4	24.7	3.0	6.4
	2	29.2	29.8	8.5	11.3
	3	44.0	37.1	9.2	12.8
	4	46.5	45.3	22.0	19.1
	5	54.6	50.2	24.1	25.5
Σ Oligosaccharides					
Digestion time (min)		**GOS-1**	**GOS-2***	**GOS-3**	**OsLu**
	0	0	0	0	0
	1	15.7	15.1	6.6	3.9
	2	24.7	19.5	10.9	10.5
	3	34.9	26.4	12.9	13.2
	4	41.0	31.2	23.0	19.0
	5	50.1	34.9	27.1	22.8

Lactose/Lactulose		**Lactose**	**Lactose**	**Lactose**	**Lactulose**		
Digestion time (min)		**GOS-1**	**GOS-2**	**GOS-3**	**OsLu**	**Lactose**	**Lactulose**
	0	**0**	**0**	**0**	**0**	**0**	**0**
	1	27.2	5.6	19.9	10.8	42.9	6.3
	2	30.1	11.1	35.9	18.1	65.4	12.4
	3	39.2	27.8	53.8	23.9	69.5	17.3
	4	49.7	33.3	61.0	27.6	81.4	23.9
	5	68.7	50.0	68.5	32.9	97.1	29.6

*Represents the sum of di-, tri and tetrasaccharide (GOS-2)

In vitro digestibility of galactooligosaccharides: Effect of the structural features on their intestinal degradation

Highlights (Ferreira-Lazarte et al.)

1.- Pig small intestinal brush border membrane vesicles were used for prebiotic digestion

2.- Chemical structure and monomer composition altered resistance to intestinal digestion

3.- β-1,6 linkages showed higher resistance to degradation than β-1,4 and β-1,3 linkages

4.- Oligosaccharides derived from lactulose were less hydrolysed than β-1,6-GOS

5.- Fructose presence in the molecular structure provided higher resistance to digestion

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In vitro fermentation properties of pectins and enzymatic-modified pectins obtained from different renewable bioresources

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ABSTRACT

The suitability of artichoke and sunflower by-products as renewable sources of pectic compounds with prebiotic potential was evaluated by studying their ability to modulate the human faecal microbiota *in vitro*. Bacterial populations and short-chain fatty acid (SCFA) production were measured. Reduction of the molecular weight of artichoke pectin resulted in greater stimulation of the growth of *Bifidobacterium*, *Lactobacillus* and *Bacteroides/Prevotella*, whilst this effect was observed only in *Bacteroides/Prevotella* for sunflower samples. In contrast, the degree of methoxylation did not have any impact on fermentability properties or SCFA production, regardless of the origin of pectic compounds. Although further *in vivo* studies should be conducted, either pectin or enzymatically-modified pectin from sunflower and artichoke by-products might be considered as prebiotic candidates for human consumption showing similar ability to promote the *in vitro* growth of beneficial gut bacteria as compared to well-recognized prebiotics such as inulin or fructo-oligosaccharides.

1. Introduction

One of the most complex polysaccharides that exist in the cell wall of all higher plants is pectin (Kačuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). Pectin is not a single structure and comprises of a family of plant cell wall polysaccharides that contain galacturonic acid (GalA) linked at α-1,4 positions. It mainly consists of a GalA-rich backbone, known as homogalacturonan (HG ≈ 65%) which is partially methyl-esterified in C-6 and O-acethyl-esterified in positions 2 and 3 (Mohnen, 2008). Rhamnose residues interrupt the HG structure to form rhamnogalacturonan I (RG-I ≈ 20–35%) which is based on a backbone consisting of a repeating disaccharide of GalA and rhamnose residues. In addition, some rhamnose residues may contain sidechains consisting of α-L-arabinose and/or β-D-galactose (arabinans, galactans and arabinogalactans). RG-II constitutes ≈ 2–10% of pectin and is the most complex, but is also believed to be the most conserved part of pectin molecules. RG-II has a HG backbone and is branched with rhamnose and other minor sugars such as fucose, glucuronic acid and methyl-esterified glucuronic acid among other rare carbohydrates such as apiose, 2-*O*-methylxylose, and 2-*O*-methylfucose (Holck, Hotchkiss, Meyer, Mikkelsen, & Rastall, 2014; Noreen et al., 2017).

The biological effects of pectins have been mainly studied on *in vitro* assays and they are highly fermentable dietary fibres. Furthermore, pectic-oligosaccharides (POS) have been proposed as a new class of prebiotics capable of exerting a number of health-promoting effects (Olano-Martin, Gibson, & Rastall, 2002). These benefits include a desirable fermentation profile in the gut (Gómez, Gullón, Yáñez, Schols, & Alonso, 2016), potential *in vitro* anti-cancer properties (Maxwell et al., 2015), potential for cardiovascular protection (Samuelsson et al., 2016), as well as antibacterial, anti-inflammatory and antioxidant properties, among others (Míguez, Gómez, Gullón, Gullón, & Alonso, 2016). Nevertheless, the details of the underlying mechanisms are still largely unknown and additional studies are needed on the structure-function interrelationship, as well as on the claimed effects caused by POS in humans (Gullón et al., 2013).

Apart from POS, whose degree of polymerization range from 3 to 10, during the past few years there has been a flourishing interest towards pectin derivatives, especially the so-called “modified pectins” (MP), a term standing for pectin-derived, water-soluble polysaccharide of lower molecular weight (Mw) than the original pectin and, normally, produced from citrus peel and pulp (Holck et al., 2014). These compounds can be obtained from pectins in their native form using chemical and enzymatic treatments, which produce lower Mw HG and fragments enriched in RG (Morris, Belshaw, Waldron, & Maxwell,

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2013). The break-down of pectins not only leads to modification of their physico-chemical and gelling properties (Ngouémazong, Christiaens, Shpigelman, Van Loey, & Hendrickx, 2015), but also modulation of their bioactivity (Morris et al., 2013).

There are several *in vitro* and *in vivo* studies on the ability of MP to inhibit tumour growth and metastasis (Morris et al., 2013; Nangia-Makker et al., 2002; Park et al., 2017). Citrus MP inhibits *in vitro* and *in vivo* angiogenesis in different types of cancer by blocking the association of galectin-3 to its receptors (Zhang, Xu, & Zhang, 2015). Other beneficial health properties might include the reduction of atherosclerotic lesions (Lu et al., 2017), anti-inflammatory and antioxidant properties (Popov & Ovodov, 2013; Ramachandran, Wilk, Melnick, & Eliaz, 2017) or immunostimulatory properties (Vogt et al., 2016). However, most of these studies were performed using cell cultures or in mice and extrapolation of the results to human or clinical investigations should be considered with caution.

Nonetheless, only a few recent studies have addressed the prebiotic potential of MP in terms of the fermentation properties. A slight or no increase was observed in the faecal lactobacilli count during an *in vivo* study with rats fed with citrus MP (Odun-Ayo, Mellem, & Reddy, 2017). Di et al. (2017) compared five structurally different citrus pectic samples (3 of them were POS and 2 were MP) and found that two POS and one MP exhibited bifidogenic effects with similar fermentabilities in human faecal cultures. These authors concluded that Mw and degree of methoxylation did not affect their bifidogenic properties; however, structural diversity in pectic compounds is possible as long as significant arabino- and galacto-oligosaccharide content is present. Fanaro et al. (2005) investigated the effect of acidic oligosaccharides from pectin on intestinal flora and stool characteristics in infants, showing that they were well tolerated as ingredient in infant formulae but did not affect intestinal microecology.

To the best of our knowledge, the fermentation and prebiotic properties of pectin derived from artichoke (Sabater, Corzo, Olano, & Montilla, 2018) and sunflower (Muñoz-Almagro, Rico-Rodriguez, Wilde, Montilla, & Villamiel, 2018) by-products have not been explored. In the case of artichoke, only one previous study showed a selective growth of two specific strains, *i.e. Lactobacillus plantarum* 8114 and *Bifidobacterium bifidum* ATCC 11,863 which was ascribed to the combination of its high inulin and low methoxylated pectin contents (Fissore, Santo Domingo, Gerschenson, & Giannuzzi, 2015). Also, Costabile et al. (2010) reported, in a double-blind, cross-over study carried out in healthy adults, a pronounced prebiotic effect (*i.e.*, increasing of bidifobacteria and lactobacilli) of a very-long-chain inulin derived from artichoke on the human faecal microbiota composition. The lack of knowledge of potential alternative sources of active pectic compounds for human consumption is surprising as previous studies reported that structure and composition can make a significant difference to the fermentation properties (Onumpai, Kolida, Bonnin, & Rastall, 2011). Thus, bifidogenic properties seem to highly depend on the composition and structure of pectins, with neutral sugar content and GalA:Rha ratio being critical factors (Di et al., 2017).

In this context, considering the structural diversity of pectins dependent on their origin, the aim of this study was to evaluate the effect of a variety of pectins and enzymatic-modified pectins from different sources (in particular, citrus, sunflower and artichoke) on the profile changes in human faecal microbiota population and fermentation metabolites, *i.e.* short-chain fatty acids.

2. Materials and methods

2.1. Raw material

Sunflower by-products based on heads and leftover stalks and artichoke by-products derived from external bracts, leaves and stems, were supplied by Syngenta AG and Riberebro S.L. (Spain), respectively. Prior to experiments, raw material was ground with a knife mill to particle size < 500 μm. Commercial citrus pectin (trade name Ceampectin®, ESS-4400) was kindly provided by CEAMSA (Porriño, Pontevedra, Spain).

2.2. Pectin extraction and modification

Sunflower pectin was extracted from 1 kg of dried substrate by suspending in 20 L of sodium citrate (0.7%) at 52 °C, pH 3.2 for 184 min under agitation and the residue was precipitated with ethanol and then freeze-dried (Muñoz-Almagro, Rico-Rodriguez, Wilde et al., 2018). Artichoke pectin was extracted using a cellulase from *Trichoderma reesei* (Celluclast® 1.5 L, Novozymes, Bagsvaerd, Denmark) in an orbital shaker at 50 °C, pH 5 with constant shaking (200 rpm) following the method described by Sabater et al. (2018). After hydrolysis, samples were centrifuged (1300 x *g* for 10 min at 4 °C) and supernatants were filtered through cellulose paper. Residues were washed and precipitated in 70% ethanol, centrifuged (1200 x *g*, 20 min) and then freeze-dried. Extraction yield of pectin (expressed as percentage) represents the amount of pectin extracted from 100 *g* of initial dried raw material, being 10.0% and 22.1% the obtained values for sunflower and artichoke pectin, respectively.

The extracted sunflower and artichoke pectins, as well as the commercial citrus pectin were then subjected to an enzymatic treatment using a commercial cellulase from *Aspergillus niger* (Sigma Aldrich, Steinheim, Germany) with pectinolytic activity to reduce their Mw. Then, the resulting material was transferred to a continuous membrane reactor to separate the modified pectin from oligosaccharides and free sugars formed (Olano-Martin, Mountzouris, Girbson, & Rastall, 2001). The reactor consisted of an ultrafiltration dead-end stirred cell (model 8000, Amicon, Watford, U.K.) where the substrate was added and then pushed from a pressurized feed tank filled with water at a rate matching the permeate flow rate. All filtrations were carried out with an Ultracel® ultrafiltration disk membrane, with a Mw cut-off (MWCO) of 3 kDa and a diameter of 76 mm as determined by the manufacturers. Checking of absence of low Mw carbohydrates in the ultrafiltered samples was accomplished by the analysis of the resulting retentates and permeates by SEC-ELSD following the method described in Section 2.3.2. All pectin and MP samples were free from monosaccharides, as well as oligosaccharides below 10 kDa (Fig. 1).

2.3. Characterisation of pectin and enzymatic-modified pectin samples

2.3.1. Monosaccharide analysis

Monosaccharide analysis was performed after the acid hydrolysis of samples with 2 M trifluoroacetic acid (TFA) at 110 °C for 4 h. After that, released monosaccharides were analysed by gas chromatography (GC) in an Agilent Technologies gas chromatograph (7890 A) equipped with a flame ionisation detector (FID). Prior to GC analysis, trimethylsilyl oximes (TMSO) of monosaccharides were formed (Cardelle-Cobas, Martínez-Villaluenga, Sanz, & Montilla, 2009). 500 μL of hydrolysed samples were evaporated to remove the acid and then 400 μL of phenyl-β-glucoside (0.5 mg/mL) used as internal standard (IS) were added. Afterward, the mixture was dried at 40 °C in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). Sugar oximes were formed by adding 250 μL hydroxylamine chloride (2.5%) in pyridine and heating the mixture at 70 °C for 30 min. Subsequently, the oximes obtained in this step were silylated with hexamethyldisylazane (250 μL) and TFA (25 μL) at 50 °C for 30 min. Derivatisation mixtures were centrifuged at 6700 x *g* for 2 min and supernatants were injected in the GC-FID.

Analyses were carried out using a DB-5HT capillary column (15 m x 0.32 mm x 0.10 μm, J&W Scientific, Folson, California, USA). Nitrogen was used as carrier gas at a flow rate of 1 mL/min. Injector and detector temperatures were 280 and 385 °C, respectively. The oven temperature was programmed from 150 to 380 °C at a heating rate of 1 °C/min until 165 °C and then up to 300 °C at a heating rate of 10 °C/min. Injections were made in the split mode (1:5).

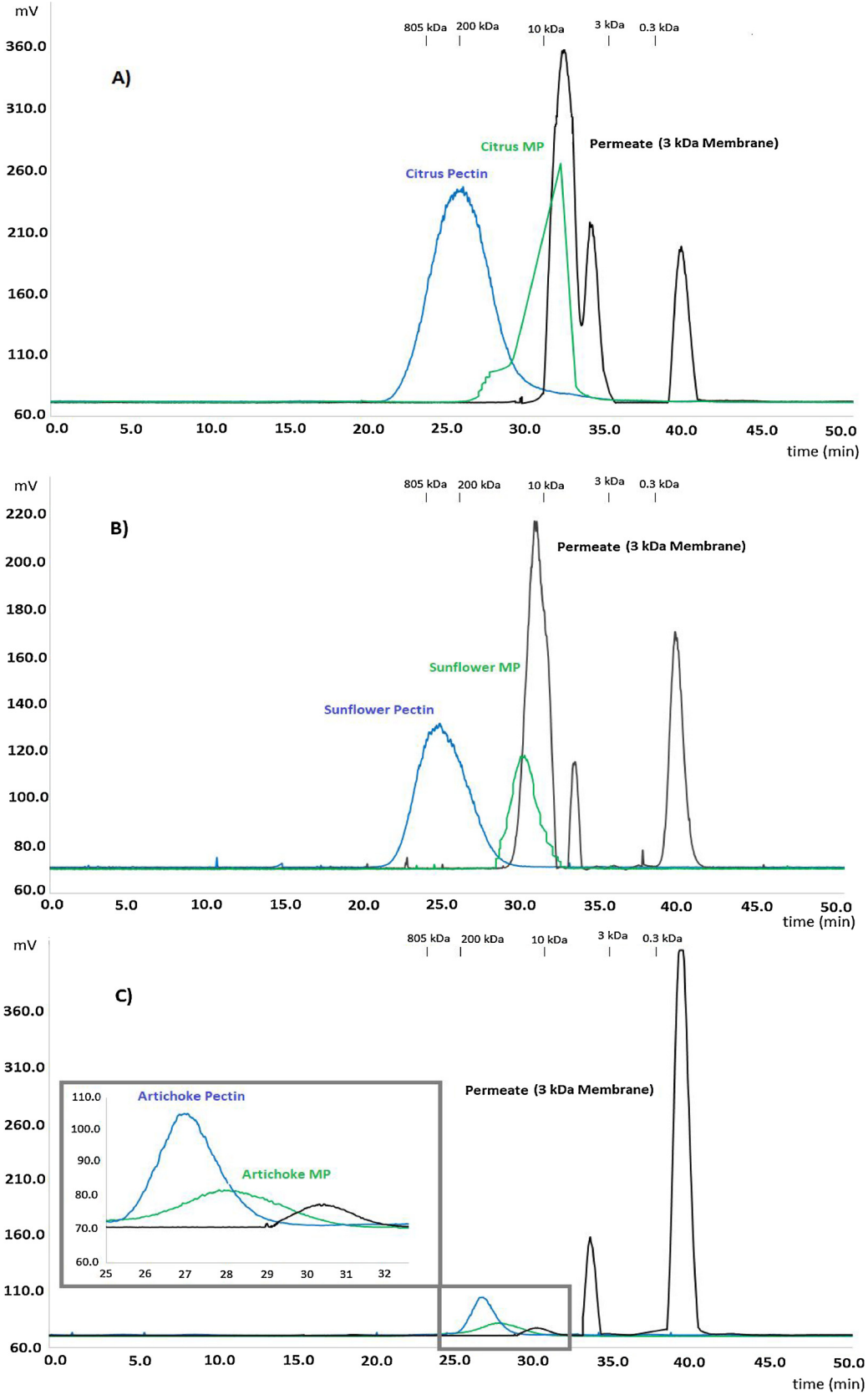

Fig. 1. SEC-ELSD profiles of pectins (blue), enzymatic-modified pectins (MP) (green), and corresponding ultrafiltrated permeates (black) derived from A) citrus, B) sunflower, and C) artichoke sources. Elution positions of standard polysaccharide polymers (pullulans) are indicated by arrows (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Data acquisition and integration were done using Agilent ChemStations software (Wilmington, DE, USA). Response factors were calculated after duplicate analysis of standard solutions (glucose, mannose, rhamnose, arabinose, galactose, GalA and xylose) over the expected concentration range in samples, (0.01–2 mg) and IS (0.2 mg).

2.3.2. Estimation of the molecular weight (Mw)

Estimation of Mw was carried out by Size Exclusion Chromatography (SEC) according to the method described by (Muñoz-Almagro, Rico-Rodriguez, Villamiel, & Montilla, 2018). The analysis was performed on a LC Agilent Technologies 1220 Infinity LC System 1260 (Agilent Technologies, Germain), equipped with two consecutive TSK-GEL columns (G5000 PW_{XL}, 7.8 × 300 mm, particle size 10 µm, G2500 PW_{XL}, 7.8 × 300 mm, particle size 6 µm) connected in series with a TSK-Gel guard column (6.0 mm × 400 mm) (Tosoh Bioscience, Stuttgart, Germany). Samples (20 µL) were eluted with 0.1 M NaCl at a flow rate of 0.5 mL/min for 50 min at 30 °C. The eluent was monitored with an Evaporative Light Scattering Detector (ELSD) (Boeblingen, Germain) at 30 °C. Pullulans of Mw 805, 200, 10, 3 and 0.3 kDa were used as standards to calibration. All the Mw values specified were weight-average.

2.3.3. Estimation of the degree of methoxylation

Degree of methoxylation of samples was determined by Fourier transform infrared spectroscopy (FTIR). KBr discs were prepared mixing the pectin and enzymatic-modified pectin samples with KBr (1:100) and pressing. FTIR spectra Bruker IFS66v (Bruker, US) were collected in absorbance mode in the frequency range of 400-4000 cm^{-1}, at a resolution of 4 cm^{-1} (mid infrared region) with 250 co-added scans. The degree of methoxylation was determined as the average of the ratio of the peak area at 1747 cm^{-1} (COO-R) and 1632 cm^{-1} (COO-) as previously described (Singthong, Cui, Ningsanond, & Douglas Goff, 2004).

2.4. Determination of in vitro fermentation properties and prebiotic activity

2.4.1. Faecal inocula

Faecal samples from five healthy adults (2 males, 3 females, mean age of 30.6 ± 4.2 years old) who had not consumed prebiotic or probiotic products, nor had received antibiotic treatment within 3 months before study were obtained *in situ*. Samples were kept in an anaerobic cabinet and used within a maximum of 15 min after collection.

Faecal samples were diluted (10% w/w) in anaerobic phosphate-buffered saline (PBS; 0.1 mol/L, pH 7.4, Oxoid, Basingstoke, UK) and homogenised in a stomacher (Stomacher 400, Seward, UK) at normal speed for 2 min.

2.4.2. In vitro batch fermentations

Sterile stirred batch culture fermentation systems were set up and aseptically filled with a volume of sterile, basal medium: (per litre) 2 g peptone water, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4{\cdot}7H_2O$, 0.01 g $CaCl_2{\cdot}6H_2O$, 2 g $NaHCO_3$, 2 mL Tween 80, 0.05 g haemin, 10 µL vitamin K1, 0.5 g L-cysteine HCl, 0.5 g bile salts and 4 mL resazurin (0.25 g/L). Medium was sterilised at 120 °C for 30 min before aseptically dispensing into the sterile fermenters. Sterile stirred fermenters were filled with 9 mL of autoclaved basal medium and were gassed overnight by constant sparging oxygen-free nitrogen to maintain anaerobic conditions. 100 mg of substrates were added (final concentration of 1% (w/v)) to the respective fermentation just prior to the addition of the faecal inoculum (1 mL). The temperature was maintained at 37 °C using a water jacket and the pH was maintained between 6.7 and 6.9 using an automated pH controller (Fermac 260; Electrolab, Tewkesbury, UK). The batch cultures were run for a period of 48 h and samples were taken from each vessel at 0 and 24 h for bacterial enumeration by fluorescent *in situ* hybridisation (FISH) and at 0, 10, 24, 36 and 48 h for SCFA by GC-FID. 3 extra vessels with inulin, fructooligosaccharides (FOS) and no added carbohydrate source were also included as positive and negative control, respectively.

2.4.3. Short-chain fatty acid (SCFA) analysis

Before chemical analysis, samples from each fermentation time were centrifuged at 13,000 x *g* for 10 min to obtain the supernatant. The clear solutions were kept at −20 °C until analysis. SCFA analysis was carried out using GC-FID based on the method described by (Richardson, Calder, Stewart, & Smith, 1989). Before analysis, samples were thawed on ice and then vortexed. After that, 400 µL of each sample were taken into a glass tube and 25 µL of 2-ethylbutyric acid (0.1 M) (IS) was added. Following that, 250 µL of concentrated HCl and 1.5 mL of diethyl ether were added and the solution was mixed 1 min and centrifuged 10 min at 2000 x *g*. 400 µL of the upper layer (ether layer) was transferred to a GC screw-cap vial and 50 µL of *N*-(*tert*-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) was added and leave 72 h to produce fully derivatisation.

A 5890 Series II Gas Chromatograph (Hewlett Packard) fitted with a Rtx-1 10 m x 0.18 mm column with a 0.20 µm coating (Crossbond 100% dimethyl polysiloxane; Restek) was used for analysis. Helium was used as carrier gas at a flow rate of 0.7 mL/min. Injector and detector temperatures were 275 °C. Oven temperature was programmed from 63 °C for 3 min and then heated to 190 °C at a heating rate of 3 °C/min and held at 190 °C for 3 min. Injections were made in the split mode (100:1). SCFA standards analysis was also carried out to quantify concentrations of all compounds.

2.4.4. Enumeration of bacterial populations

Enumeration of the target faecal bacteria groups was achieved by FISH with fluorescently labelled 16S rRNA probes according to the method described by (Wagner, Hornt, & Daims, 2003). Briefly, 375 µL aliquots were obtained from each fermenter and were mixed with 1.125 mL 4% (w/v), ice-cold paraformaldehyde and fixed for 4–10 h at 4 °C. Fixed cells were then centrifuged at 13,000 x *g* for 5 min and washed twice on 1 mL cold filter-sterilised PBS (0.1 M). The washed cells were then resuspended in 150 µL PBS and 150 µL of absolute ethanol (99%) and stored at −20 °C until analysis.

To obtain an appropriate number of fluorescent cells in each field of view of the microscope, samples to hybridise were then diluted in a suitable volume of PBS with 1% (v/v) of sodium dodecyl sulphate, and 20 µL of the dilution was added to each well of a six-well polytetrafluoroethylene/poly-L-lysine-coated slide (Tekdon Inc., Myakka City, USA). Samples were dried at 48–50 °C for 15 min in a desktop plate incubator and dehydrated in an alcohol series (50, 80 and 96% (v/v) ethanol, 2 min each) and placed again at 48–50 °C to evaporate the excess of ethanol before adding the hybridisation solution. 50 µL of hybridisation solution (per 1 mL; 5 M NaCl 180 µL, 1 M Tris/HCl 20 µL, ddH_2O 799 µL, 1 µL SDS 10% (w/v) and 100 µL of probe) was added to each well and left to hybridise for 4 h in a microarray hybridisation incubator (Grant-Boekel, UK) at 46–50 °C depending on the probe. After hybridisation, slides were washed in 50 mL washing buffer (5 M NaCl 9 mL, ddH_2O 40 mL and 1 M Tris/HCl 1 mL) for 15 min and dipped in cold distilled water for 2–3 seconds. Slides were then dried with compressed N_2 and a drop of PVA-DABCO antifade (polyvinyl alcohol mounting medium with 1,4-diazabicyclo (2.2.2) octane) was added onto each well. A coverslip (20 mm, thickness no. 1; VWR) was placed on each slide and cell numbers of microorganisms were determined by direct counting under an epifluorescence microscope (Eclipse 400; Nikon, Surrey, UK) with Fluor 100 lens. A total of 15 random fields of view were counted for each well.

The oligonucleotide probes used and conditions for each one are detailed in Table 1. These probes were selected to account for major bacterial groups in the Actinobacteria (Bif164), Bacteroidetes (Bac303), and Firmicutes (Lab158, Erec482, Chis150) phyla.

Table 1
Oligonucleotide probes used in this study for FISH enumeration of bacteria.

Probe	Specificity	DNA Sequence (5' to 3')	Temperature (°C)		Reference
			HB*	WB*	
Bac303	Most *Bacteroidaceae* and *Prevotellaceae*. some *Porphyromonadaceae*	CCA ATG TGG GGG ACC TT	46	48	Manz, Amann, Ludwig, Vancanneyt, and Schleifer, (1996)
Bif164	*Bifidobacterium spp.*	CAT CCG GCATTA CCA CCC	50	50	Langendijk et al. (1995)
Chis150	Most of the *Clostridium histolyticum* group (Clostridium cluster I and II)	TTA TGC GGT ATT AAT CT(C/T) CCT TT	50	50	Franks et al. (1998)
Erec482	Most of the *Clostridium coccoides-Eubacterium rectale* group (Clostridium cluster XIVa and XIVb)	GCTTCT TAGTCA (A/G)GT ACC G	50	50	Franks et al. (1998)
Lab158	*Lactobacillus; Enterococcus*	GGT ATT AGC A(C/T)C TGT TTC CA	50	50	Harmsen, Elfferich, Schut, and Welling, (1999)

*HB: hybridisation buffer; WB: washing buffer.

2.5. Statistical analysis

Statistical analysis was performed using SPSS for Windows, version 23.0. One-way analysis of variance (ANOVA) and Tukey's *post hoc* test was used to determine significant differences among the bacterial group populations and organic acid concentrations among the different substrates. Differences were considered significant at $p < 0.05$ (n = 5).

3. Results and discussion

The yields of extraction of pectin from artichoke (22.1%) and sunflower by-products (10.0%) were in line with those obtained for other well-established sources of pectin, such as citrus peel (Kurita, Fujiwara, & Yamazaki, 2008), lime peel (Dominiak et al., 2014), apple pomace (Wikiera, Mika, Starzyńska-Janiszewska, & Stodolak, 2015) or passion fruit peels (Liew, Chin, Yusof, & Sowndhararajan, 2016), suggesting their potential use as renewable pectin sources.

3.1. Characterisation of pectin and enzymatic-modified pectin samples

Pectins from different sources (that is, citrus, artichoke and sunflower) and their enzymatic modified polysaccharides (modified pectin (MP)) were evaluated in this study. Neutral sugars and GalA content, average degree of methoxylation and average estimated Mw are included in Table 2. The GalA:Rha ratio displayed in the table shows the number of GalA residues per Rha residue, giving an indication of the RG-I backbone respect to HG content. Thus, a lower value shows a compound richer in RG-I chains. Ara:Rha and Gal:Rha ratios indicate the number of neutral sugar residues attached to the RG-I backbone.

As expected, GalA was the major monosaccharide residue in all pectic samples, ranging from 46.5% (w/w) to 88.1% (w/w). The lowest values of GalA content were observed in those samples which had the highest values of rhamnose content. In consequence, the GalA:Rha ratio indicated that citrus MP, artichoke pectin, artichoke MP and citrus pectin were the most enriched samples in RG-I as compared to sunflower samples, which were the most enriched in HG structure according to the monomeric composition (27.4 and 24.1 for GalA:Rha ratio for sunflower pectin and sunflower MP, respectively). Instead, artichoke pectin and MP presented high amounts of arabinose, surpassing rhamnose content, which could be indicative of a highly enriched structure in arabinan and arabinogalactan branches to the RG-I chains. The amount of rhamnose and arabinose with respect to GalA may also indicate the substitution of the rhamnogalacturonan branching along the HG with arabinan and arabinogalactan structures (Manderson et al., 2005; Yuliarti, Goh, Matia-Merino, Mawson, & Brennan, 2015). The high content of arabinose and GalA determined in artichoke samples support the data obtained in previous studies (Femenia, Robertson, Waldron, & Selvendran, 1998; Sabater et al., 2018). Galactose content in all samples was higher than other neutral sugars, with the exception of arabinose in artichoke pectin, which may also indicate the presence of galactose-based oligosaccharides branched to the HG backbone. Xylose that can be present in more complex structural features of pectin, such as RG-II regions or arabinoxylans and xylogalacturonan (Maxwell, Belshaw, Waldron, & Morris, 2012), ranged from 0.9% to 2.3%. Lastly, glucose (from 0.9% to 16.7%) and mannose (from 0.1% to 2.4%) were found in all samples and they could likely derive from non-pectic polysaccharides extracted in minor amounts together the target pectins, such as xyloglucan, hemicellulose, and/or cellulose (Sabater et al., 2018; Wang et al., 2016; Yapo, 2009).

In both artichoke samples the degree of methoxylation was the

Table 2
Chemical characterisation of pectins and enzymatic-modified pectins from different renewable bioresources.

Sample	Monosaccharide (%*)							Average Mw (kDa)	GalA:Rha	Ara:Rha	Gal:Rha	Average degree of methoxylation (%)
	Xylose	Arabinose	Rhamnose	Galactose	Mannose	Glucose	Galacturonic acid					
Citrus Pectin	0.9 ± 0.0	3.5 ± 0.0	5.8 ± 0.0	20.2 ± 0.1	1.4 ± 0.0	1.8 ± 0.0	66.5 ± 0.2	800-100	11.52	0.61	3.50	70.7
Citrus MP	1.3 ± 0.2	3.7 ± 0.2	9.8 ± 0.1	14.0 ± 0.3	2.4 ± 0.3	13.3 ± 0.3	55.6 ± 0.6	12.0-10.0	5.70	0.38	1.44	14.2
Sunflower Pectin	2.2 ± 0.1	1.1 ± 0.0	3.2 ± 0.4	4.3 ± 0.0	0.1 ± 0.0	0.9 ± 0.0	88.1 ± 0.9	800-100	27.39	0.35	1.35	45.7
Sunflower MP	0.9 ± 0.0	2.3 ± 0.0	3.2 ± 0.1	12.2 ± 0.0	1.3 ± 0.0	1.8 ± 0.0	78.2 ± 0.5	12.5	24.13	0.71	3.77	17.0
Artichoke Pectin	1.1 ± 0.1	18.9 ± 0.6	7.6 ± 0.1	8.2 ± 0.3	1.0 ± 0.3	16.7 ± 0.7	46.5 ± 0.6	> 500	6.13	2.50	1.09	8.9
Artichoke MP	2.3 ± 0.2	10.7 ± 0.1	5.4 ± 0.0	21.1 ± 0.0	1.2 ± 0.0	3.9 ± 0.0	55.5 ± 0.8	300-80	10.34	1.99	3.94	8.5

Analysis were carried out at least in duplicate (n = 2).
*Monosaccharide content (%) is referred regarding the total carbohydrate measured on each sample.

lowest (8.9 and 8.5% for pectin and MP, respectively), whereas MP samples from citrus and sunflower had moderately higher values (14.2 and 17.0%, respectively) and citrus and sunflower pectin had the highest data of all samples with 70.7% and 45.7% of degree of methoxylation, respectively. This behaviour could be ascribed to the pectin methyl esterase activity of the enzyme employed to produce the corresponding MP.

On the other hand, all resulting MP showed a reduction of the Mw as compared to their respective pectin due to the polygalacturonase enzyme activity, which was concomitant with a decrease in GalA and an increase in RG-I to HG. However, modified artichoke pectin showed a decrease in arabinose which led to a higher relative content of GalA compared to its parent pectin. The initial high content of arabinose observed in artichoke pectin could be related to the resulting high Mw of artichoke MP following enzymatic treatment. It is well known that arabinose is present in pectin as arabinan side chains and, consequently, a high degree of branching may create steric hindrance impairing the efficient cutting of the main chain composed by GalA. The decrease in Mw was correlated to the diminution of degree of methoxylation observed in citrus and sunflower samples. It is interesting to note that citrus and sunflower MP exhibited a Mw of 10–12.5 kDa which is in line with other modified pectins obtained from citrus (10 kDa) that have shown to be effective supplements in the treatment of cancer and other diseases (Morris et al., 2013). Artichoke MP showed a small decrease in this parameter which is in accordance with its high Mw, as shown in Fig. 1.

3.2. In vitro fermentation

3.2.1. Bacterial population changes during in vitro fermentation

Changes in the human faecal bacterial populations during the *in vitro* fermentation with the different pectins and enzymatic-modified pectins after 24 h are shown in Table 3. A significant increase ($p < 0.05$) of *Bifidobacterium* (Bif164) population for all carbohydrate samples was observed after 24 h of fermentation. It is well known that oligosaccharides deriving from pectins have bifidogenic activities, however there are also studies that have demonstrated a bifidogenic effect in intact pectins suggesting a potential role of this polysaccharide as a prebiotic (Gómez et al., 2016; Yang, Martínez, Walter, Keshavarzian, & Rose, 2013). In our study, numerical increases up to 0.79–1.19 $\log_{10}$ in population were determined. Some authors indicated that increments of 0.5–1.0 $\log_{10}$ in bifidobacteria could be considered as a major shift in the gut microbiota towards a potentially healthier composition of intestinal microbiota (Kolida & Gibson, 2007). Thus, all pectic samples could be considered bifidogenic under the studied conditions. Remarkably, artichoke MP was the substrate, which promoted the significantly highest growth in bifidobacteria among all assayed samples, including positive controls as inulin and FOS which in turn showed a similar bifidobacterial growth as compared to sunflower and citrus samples. This fact could be attributed to the high combined content of arabinose and galactose found in artichoke MP (Table 2) according to previous studies reporting a correlation between arabinose and galactose content with bifidogenic properties (Di et al., 2017; Manderson et al., 2005; Onumpai et al., 2011). Moreover, a positive effect of the decrease of Mw in pectin on its ability to promote bifidobacteria growth was observed for citrus and artichoke sources since their MP derivatives exhibited a significant ($p < 0.05$) increase as compared to unmodified pectin (9.63 *vs* 9.42 $\log_{10}$ for citrus and 9.82 *vs*. 9.50 for artichoke), whereas sunflower pectin and MP presented a statistically identical bifidogenic activity. Evidently, there was not any significant increase during fermentation of negative controls, confirming the suitability of these substrates as a carbon source for the metabolism of bifidobacteria. The degree of methoxylation did not have impact on the bifidogenic properties. More specifically, sunflower samples had different value of this parameter with the same bifidogenic activity and artichoke samples had almost the same one with different bifidogenic activity.

The second highest increase (up to 0.56–0.93 $\log_{10}$) was observed in *Bacteroides/Prevotella* (Bac303) population. This general increase is explained by the fact that *Bacteroides* species are major carbohydrate-degrading organisms in the gut and have the capacity to degrade diverse plant polysaccharides, including pectins (Dongowski, Lorenz, & Anger, 2000; Flint, Scott, Duncan, Louis, & Forano, 2012; Onumpai et al., 2011). Indeed, many *Bacteroides* strains from human faeces can produce pectinolytic enzymes, including polygalacturonase and pectin methylesterase (Dekker & Palmer, 1981; Jensen & Canale-Parola, 1986). Therefore, *Bacteroides* can be involved in cross-feeding with *Bifidobacteria* by releasing breakdown products of pectin or MP which might be utilised by the latter, thus, promoting their growth. Inulin, FOS and artichoke MP samples exhibited the highest increase in

Table 3
Bacterial populations (log10 cells per ml) enumerated by FISH at 0 and 24 h of *in vitro* fermentation with Inulin, FOS, citrus pectin, citrus modified pectin (MP), sunflower pectin, sunflower MP, artichoke pectin and artichoke MP.

Probe/Strain	Time point (h)	Bacterial concentration (log10 cells/mL)								
		Control	Inulin	FOS	Citrus Pectin 800-100 kDa	Citrus MP 10.0 – 12.0 kDa	Sunflower Pectin 800-100 kDa	Sunflower MP 12.50 kDa	Artichoke Pectin > 500 kDa	Artichoke MP 300-80 kDa
Bif164	**0**	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)	8.63 (0.08)
	24	8.75 (0.03)^{a}	9.52 (0.15)^{bc,1}	9.48 (0.05)^{bc,1}	9.42 (0.06)^{b,1}	9.63 (0.04)^{cd,1}	9.72 (0.12)^{cd,1}	9.74 (0.06)^{cd,1}	9.50 (0.14)^{bc,1}	9.82 (0.13)^{d,1}
Bac303	**0**	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)	8.46 (0.08)
	24	8.59 (0.08)^{a}	9.36 (0.05)^{ef,1}	9.39 (0.04)^{f,1}	9.05 (0.08)^{bc,1}	9.06 (0.03)^{bc,1}	9.02 (0.09)^{b,1}	9.19 (0.07)^{cd,1}	9.23 (0.11)^{de,1}	9.45 (0.04)^{f,1}
Lab158	**0**	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)	8.35 (0.12)
	24	8.38 (0.07)^{a}	9.05 (0.04)^{de,1}	8.98 (0.03)^{cd,1}	8.65 (0.06)^{b,1}	9.04 (0.03)^{d,1}	9.05 (0.02)^{d,1}	8.98 (0.09)^{cd,1}	8.92 (0.05)^{c,1}	9.17 (0.05)^{e,1}
Erec482	**0**	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)	8.50 (0.07)
	24	8.51 (0.04)^{a}	8.97 (0.11)^{bc,1}	9.08 (0.11)^{c,1}	9.02 (0.05)^{bc,1}	9.06 (0.06)^{c,1}	8.83 (0.11)^{b,1}	8.97 (0.07)^{bc,1}	8.95 (0.06)^{bc,1}	9.01 (0.11)^{bc,1}
Chis150	**0**	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)	8.29 (0.05)
	24	8.35 (0.04)^{a}	8.77 (0.06)^{b,1}	8.72 (0.03)^{b,1}	8.70 (0.09)^{b,1}	8.73 (0.03)^{b,1}	8.77 (0.01)^{b,1}	8.70 (0.02)^{b,1}	8.72 (0.04)^{b,1}	8.70 (0.06)^{b,1}

A control sample without carbohydrate source is also included. Experiments were carried out in batch cultures systems inoculated with faecal inocula from five healthy human donors. Results shown as mean (n = 5) with the corresponding standard deviation in parentheses.
$^{a.b.c}$ Significant differences ($p < 0.05$) between substrates are indicated with different letters in the same row.
^{1} Significant difference ($p < 0.05$) from the 0 h value for each bacterial group and for the same substrate.

Bacteroides. With respect to the effect of Mw on *Bacteroides/Prevotella* growth, sunflower and artichoke MP demonstrated a significantly higher increase than their respective pectins. This difference could be attributed to the galactan chains branched to the RG-I since Gal:Rha ratio increased in both sunflower and artichoke MP after the enzymatic hydrolysis.

A significant increase in *Lactobacillus/Enterococcus* (Lab158) was also observed for all tested carbohydrate samples, with the most significant increases found in inulin and artichoke MP. Similar to *Bifidobacterium*, *Lactobacillus* is considered one of the major microbial targets for prebiotic action due to their health effects. The high increment in *Lactobacillus/Enterococcus* population following artichoke MP fermentation further established the correlation of arabinose and galactose content with the prebiotic properties. Mw did not affect sunflower samples but it seemed to have an impact on citrus and artichoke sources, in a similar manner to the behaviour observed for *Bifidobacterium* selectivity.

Clostridium coccoides/Eubacterium rectale (Erec482) showed a significant increase in all tested samples but no significant differences were found among any of the carbohydrate substrates including inulin and FOS. Increase in *Eubacterium rectale* is of particular interest due to its ability to produce butyrate (Manderson et al., 2005). Di et al. (2017) reported an increase of Erec482 numbers when testing a citrus MP of similar Mw (9.2 kDa), although they did not find a positive correlation with the determined butyrate concentrations. In the same way, Chen et al. (2013) reported enhanced *Eubacteria* growth on apple pectin compared to the respective POS, suggesting that the Mw was not a relevant factor. In our work, similar behaviour was observed since all pectic samples resulted in a significant stimulation of the butyrate producing bacteria groups (Erec482) and no differences were found between samples with different Mw or origin.

Clostridium histolyticum (Chis150) population displayed the lowest changes in all cases, leading to a rather moderate increase (lower than 0.5 $\log_{10}$) after 24 h of fermentation. No significant differences among any substrates were observed after fermentation. In general, *Clostridium* species are considered as potentially harmful bacteria, so in this way, all pectic samples induced a favourable behaviour.

3.2.2. Short-chain fatty acids (SCFA) production

Acetate, propionate, butyrate and total SCFA formation was analysed throughout the fermentation in batch cultures (Table 4). Total SCFA concentration increased strongly during the first 10 or 24 h of fermentation in all tested substrates. In general terms, neither the degree of methoxylation nor Mw of pectin samples had an influence on the SCFA production, as reflected by the values contained in Table 4.

Acetate was the most abundant SCFA, followed by propionic and butyric acids in all substrates. Formation of acetate has been related to an enhancement of the ileal motility, a protection against genotoxic agents and pathogens and an increase of colonic blood (Hong et al., 2005). In our study, the only significant differences found between samples after 48 h of analysis were with artichoke and citrus MPs. Results demonstrated a sharp increase of this compound in the first 10 h of fermentation. Although it is challenging to attribute a particular fermentation end-product to a specific bacterial group in a mixed culture system, overall the increase in acetate is in agreement with the dynamics of the microbial populations, since all samples promoted the growth of *Bifidobacterium* and *Lactobacillus* (Table 3), which are acetate producers. Additionally, these end-products may serve as substrates for other bacteria due to metabolic cross-feeding (Belenguer et al., 2006). Acetate is generated by many bacterial groups that inhabit the colon, with approximately one-third of the product coming from reductive acetogenesis (Miller & Wolin, 1996). In contrast, bacterial groups that form propionate and butyrate are specialised and are of particular interest in terms of their beneficial effects. The main propionate-producing bacteria in the human colon are *Bacteroides* and *Clostridium* whereas butyrate production is related to bacterial groups such as *Clostridium histolyticum* (clusters I, II, IV, XIVa, XV and XVI) and *Eubacterium rectale*.

An increase in propionate concentration was seen in all samples after 48 h of fermentation, whereas fermentation of inulin and FOS resulted in the highest increase among all samples. Similarly to acetate, the high variability found among the five donors meant that propionate differences between all samples were not considered statistically significant ($p > 0.05$) during the first 24 h of fermentation. However, the increase in this end-product is in good agreement with the increase in *Bacteroides* population displayed in Table 3. Propionate has also been shown to exert beneficial effects on host health, such as reduction of food intake and enhancement of satiety *via* augmentation of the satiety hormone leptin (Zeng, 2014), and a protective role against carcinogenesis through the decrease in human colon cancer cell growth *via* hyperacetylation of histone proteins and stimulation of apoptosis (Hinnebusch, Meng, Wu, Archer, & Hodin, 2002; Jan et al., 2002).

Butyrate production resulted in a significant increase in all samples after 24 h of fermentation. FOS and inulin showed the highest increase after 48 h of fermentation, although non-significant differences were observed among all substrates due to the high inter-individual variability (Table 4). The low but significant increase in butyrate levels are in accordance with the increase of Erec482 and Chis150 numbers which also include some of the major butyrate-produces (*Eubacterium rectale* and *Clostridium histolyticum)*. Although acetate, propionate and butyrate are all metabolised to some extent by the epithelium to provide energy, butyrate plays a critical role in maintaining colonic health and moderating cell growth (Zeng, 2014). Compared to acetate and propionate, butyrate exhibits strong anti-inflammatory properties, likely mediated by inhibition of TNF-α production, NF-κB activation, and IL-8, -10, -12 expression in immune and colonic epithelial cells and a protective role against colon cancer (Bailón et al., 2010; Zeng, 2014).

4. Conclusions

Findings in this work highlight the suitability of artichoke and sunflower by-products as renewable sources of bioactive pectic compounds since the reported yields were within the range observed for other well-established pectin sources. To the best of our knowledge, this is the first evidence of prebiotic potential of pectic compounds from sunflower and artichoke and also supports the important role played by the arabinose-rich rhamnogalacturonic acids in stimulating *Bifidobacteria*. A positive effect of decreasing molecular weight on fermentation properties was found in artichoke and citrus sources since their respective enzymatically-modified pectins promoted significantly higher growth in *Bifidobacterium* and *Lactobacillus* than the corresponding unmodified pectin. In the case of sunflower, this behaviour was only observed in *Bacteroides/Prevotella*, which also grew to significantly higher population levels on artichoke MP as compared to the unmodified pectin. No significant effects of the Mw of pectin samples on SCFA production were observed, although this could be due to the high inter-individual variability observed in acetate, propionate and butyrate formation. Likewise, the degree of methoxylation did not have any significant impact on the fermentability nor SCFA production, regardless the origin of the pectic compounds.

To conclude, although further *in vivo* studies should be conducted, our data reveal that either pectin or enzymatically-modified pectin from sunflower and artichoke by-products might be considered as efficient prebiotic candidates for human consumption showing similar ability to promote the *in vitro* growth of beneficial gut bacteria as *Bifidobacterium* and *Lactobacillus* in comparison to well-recognized prebiotics as inulin and FOS.

Declarations of interest

None.

Table 4
SCFA concentrations (mM) determined by GC-FID at 0, 10, 24, 36 and 48 h on *in vitro* fermentations with Inulin, FOS, citrus pectin, citrus modified pectin (MP), sunflower pectin, sunflower MP, artichoke pectin and artichoke MP.

SCFA	Time point (h)	Mean SCFA concentration (mM) in substrate								
		Control	Inulin	FOS	Citrus Pectin 800-100 kDa	Citrus MP 10.0 – 12.0 kDa	Sunflower Pectin 800-100 kDa	Sunflower MP 12.50 kDa	Artichoke Pectin > 500 kDa	Artichoke MP 300-80 kDa
Acetate	**0**	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)	3.80 (1.55)
	10	12.28 (3.30)^{a,1}	36.99 (6.60)^{b,1}	54.64 (15.10)^{bcd,1}	61.65 (11.81)^{cd,1}	68.49 (6.19)^{d,1}	50.64 (9.15)^{bcd,1}	58.47 (4.13)^{cd,1}	49.35 (5.54)^{bcd,1}	42.19 (3.77)^{bc,1}
	24	21.11 (3.31)^{a,2}	62.44 (11.68)^{bcd,2}	57.89 (14.88)^{bc}	78.42 (9.02)^{cd}	78.83 (12.87)^{cd}	82.65 (11.80)^{d,2}	69.21 (10.29)^{bcd}	55.33 (1.62)^{b}	50.86 (7.81)^{b}
	36	26.18 (4.49)^{a}	65.24 (11.98)^{bc}	63.68 (10.80)^{bc}	71.18 (11.38)^{bc}	78.95 (11.71)^{c}	78.95 (11.62)^{c}	67.64 (9.27)^{bc}	55.64 (4.57)^{b}	55.60 (11.09)^{b}
	48	17.62 (3.38)^{a}	64.42 (10.55)^{bc}	63.55 (10.86)^{bc}	77.99 (14.69)^{bc}	85.40 (11.34)^{c}	78.49 (13.31)^{bc}	73.87 (10.49)^{bc}	61.00 (12.27)^{bc}	55.94 (8.95)^{b}
Propionate	**0**	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)	0.54 (0.27)
	10	2.28 (0.92)^{a}	7.58 (1.91)^{ab}	12.20 (6.90)^{b}	8.13 (3.22)^{ab,1}	11.6 (3.76)^{b,1}	6.35 (2.33)^{ab,1}	10.00 (1.10)^{ab,1}	11.35 (4.10)^{b,1}	11.27 (3.17)^{b,1}
	24	4.7 (1.26)^{a,2}	18.17 (4.69)^{b,2}	15.64 (6.76)^{b,1}	12.12 (4.58)^{ab}	16.04 (1.86)^{b}	11.82 (1.26)^{ab,2}	13.84 (0.90)^{b,2}	13.49 (3.98)^{b}	15.69 (1.37)^{b,2}
	36	4.1 (0.60)^{a}	19.51 (5.68)^{cd}	23.77 (2.89)^{d}	11.92 (4.26)^{b}	16.15 (2.26)^{bcd}	11.80 (1.47)^{b}	14.12 (1.61)^{bc}	14.55 (3.51)^{bc}	16.97 (1.62)^{bcd}
	48	2.12 (0.99)^{a}	18.41 (4.95)^{bc}	20.69 (6.22)^{c}	12.71 (3.51)^{b}	17.35 (2.12)^{bc,2}	13.60 (3.01)^{bc}	14.15 (1.82)^{bc}	14.10 (3.61)^{bc}	16.10 (2.03)^{bc}
Butyrate	**0**	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)	0.96 (0.17)
	10	1.29 (0.64)^{a}	6.57 (0.75)^{b}	3.43 (2.17)^{ab}	1.82 (1.94)^{a}	2.51 (1.36)^{a}	2.22 (1.10)^{a}	3.26 (1.52)^{ab}	2.70 (1.88)^{a}	2.74 (1.12)^{a}
	24	1.77 (1.03)^{a}	9.13 (2.22)^{b,1}	5.20 (3.28)^{ab}	4.52 (1.94)^{a,1}	4.94 (1.54)^{ab,1}	4.54 (2.19)^{a,1}	5.42 (1.59)^{ab,1}	5.30 (1.68)^{ab,1}	4.50 (0.78)^{a,1}
	36	2.31 (0.67)^{a,1}	9.66 (3.14)^{c}	7.60 (3.20)^{bc,1}	5.86 (2.26)^{abc,2}	5.35 (1.87)^{abc,2}	5.13 (1.88)^{abc}	6.23 (1.80)^{abc}	5.25 (2.22)^{abc}	4.98 (1.34)^{ab}
	48	1.06 (0.38)^{a}	9.08 (2.87)^{b}	8.40 (3.85)^{b}	4.92 (2.08)^{ab}	5.89 (1.59)^{ab}	4.78 (1.31)^{ab}	6.33 (2.61)^{b}	6.04 (3.04)^{b}	4.98 (1.87)^{ab}
Total	**0**	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)	5.26 (1.90)
	10	15.84 (4.61)^{a,1}	49.14 (13.14)^{b,1}	83.76 (17.01)^{c,1}	71.42 (12.18)^{bc,1}	82.95 (6.94)^{c,1}	59.21 (11.65)^{bc,1}	71.74 (6.46)^{bc,1}	63.89 (10.69)^{bc,1}	56.20 (7.58)^{b,1}
	24	27.83 (3.69)^{a,2}	90.30 (11.28)^{bcd,2}	84.14 (17.36)^{bcd}	94.15 (11.21)^{bcd}	102.36 (14.94)^{d}	99.01 (11.79)^{cd,2}	89.70 (9.42)^{bcd}	74.99 (3.97)^{bc}	72.42 (9.24)^{b}
	36	32.24 (4.55)^{a}	95.90 (13.77)^{b}	90.74 (15.10)^{b}	88.97 (14.71)^{b}	97.03 (18.08)^{b}	95.88 (12.16)^{b}	89.64 (10.97)^{b}	77.27 (8.63)^{b}	79.42 (13.64)^{b,2}
	48	21.21 (3.96)^{a}	90.38 (18.27)^{bc}	91.45 (11.89)^{bc}	95.63 (16.72)^{bc}	109.42 (12.10)^{c}	96.87 (13.57)^{bc}	98.84 (9.49)^{bc,2}	83.19 (17.16)^{bc}	77.02 (11.35)^{b}

A control sample without carbohydrate source was also included. Experiments were carried out in batch cultures systems inoculated with faecal inocula from five healthy human donors. Results shown as mean (n = 5) with the corresponding standard deviation in parentheses.

[a. b. c]Significant differences ($p < 0.05$) between substrates are indicated with different letters in the same row.

[1]Significant difference ($p < 0.05$) from the 0 h value for each SCFA and for the same substrate.

[2]Significant difference ($p < 0.05$) from the 10 h value for each SCFA and for the same substrate.

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Behaviour of citrus pectin during its gastrointestinal digestion and fermentation in a dynamic simulator (simgi®)

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ABSTRACT

The behaviour of citrus pectin during digestion and its potential prebiotic properties were examined using a Dynamic Gastrointestinal Simulator (simgi®) model for the human gut, which simulates processes in the stomach, small intestine, ascending, transverse and descending colon. A remarkable non-digestibility of pectin in the upper gastrointestinal tract was observed by HPLC-ELSD analysis, where ~88% of citrus pectin remained intact during its transit through the stomach and small intestine. Fermentation of pectin stimulated the growth of beneficial bacteria such as *Bifidobacterium* spp, *Bacteroides* spp and *Faecalobacterium prausnitzii*. High increases of short-chain fatty acids (SCFA) were observed, especially in acetate and butyrate concentration due to direct fermentation of pectin or by cross-feeding interaction between bacteria. This is the first study on the digestibility and fermentation of pectin carried out in a complex dynamic gastrointestinal simulator, being of special relevance the results obtained for *F. prausnitzii*.

1. Introduction

Pectins are a family of plant cell wall polysaccharides with glycan domains that contain galacturonic acid (GalA) units with α-1,4 linkages. It mainly consists of a GalA-rich backbone, known as homogalacturonan (HG ≈ 65%), of which a number of residues are methyl esterified at the C-6 position, thereby conferring a specific degree of methoxylation (DM) to the polymer. This parameter and its distribution pattern define the charge distribution over the polymer playing a major role in the dimerization of pectin chains through the formation of junction zones, either *via* cooperative Ca^{2+} complexation or at reduced water activity as well as pH, thus defining the gelation properties of pectin (Dongowski, Lorenz, & Proll, 2002; Fraeye, Duvetter, Doungla, Van Loey, & Hendrickx, 2010). Furthermore, rhamnose residues interrupt the HG structure to form rhamnogalacturonan I (RG-I ≈ 20–35%) which is based on a backbone consisting of a repeating disaccharide of [→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p*-(1→] residues. RG-I has a number of side chains attached to its backbone and the length of these chains can vary from single glycosyl to polymeric side chains of different types (1→5)-α-L-arabinans, (1→4)-β-D-galactans, arabinogalactans-I, arabinogalactans-II (Buffetto et al., 2015). RG-II constitutes ≈ 2–10% of pectin and is the most complex part of pectin, it has a HG backbone branched with rhamnose and other minor sugars such as fucose, glucuronic acid, methyl-esterified glucuronic acid, apiose, 2-O-methylxylose, and 2-O-methylfucose (Holck, Hotchkiss, Meyer, Mikkelsen, & Rastall, 2014; Lara-Espinoza, Carvajal-Millán, Balandrán-Quintana, López-Franco, & Rascón-Chu, 2018; Noreen et al., 2017). The suitability of pectins for specific applications is governed by the structural features, including molecular weight (Mw), neutral sugar content, proportion of HG:RG-I regions or the DM(Ferreira-Lazarte, Kachrimanidou, Villamiel, Rastall, & Moreno, 2018; Sila, Van Buggenhout, Duvetter, Van Loey, & Hendrickx, 2009). These factors can affect their applicability as thickeners or as gelling and stabilizing agents (Gullón et al., 2013).

Pectins, as other dietary fibers, are believed to be resilient to digestion reaching the hindgut where they are fermented by the colonic microbiota (Lunn & Buttriss, 2007). However, before they reach the colon, these heteropolysaccharides are subjected to the singular luminal environment of the upper digestive tract that can contribute to chemical and physicochemical changes affecting the rate and extent of the fermentation in the colon (Hoebler, Guillon, Fardet, Cherbut, & Barry, 1998). The intestinal degradation of pectin has been studied with substantially dissimilar results. In studies involving human subjects, Chinda et al. (2003) and Saito et al. (2005) as well as Holloway,

Abbreviations: GIT, gastro intestinal tract; POS, pectic-oligosaccharides; simgi®, SIMulator gastro-intestinal model; AC, ascending colon; TC, transverse colon; DC, descending colon; Mw, molecular weight; DM, degree of methoxylation; SCFA, short chain fatty acid

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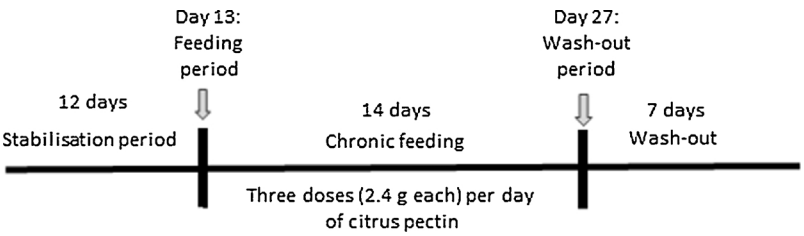

Fig. 1. Schematic representation of the experimental protocol of the simgi® trial developed to assess the gastrointestinal digestion and fermentation properties of citrus pectin (30 g/L in nutritive medium).

Tasman-Jones, and Maher, (1983) observed that around 90% and 60–85% of apple and citrus pectin, respectively, reached the terminal ileum but the procedures to evaluate total pectin were not robust enough to identify the possible structural and physical changes that take place.

However, the capability of pectins to be fermented by the intestinal microbiota it is well known, being the arabino- and galacto-oligosaccharides content one of the most important factors, even more relevant than Mw (Di et al., 2017; Onumpai, Kolida, Bonnin, & Rastall, 2011). In this sense, there are some investigations that report a better bifidogenic effect, which means a growth of *Bifidobacteria* population, of pectins and pectic-polysaccharides with higher arabino- and galacto-oligosaccharides content, over modified pectin and pectic-oligosaccharides (POS) with lower Mw (Di et al., 2017; Ferreira-Lazarte et al., 2018).

Most of these studies are *in vitro* and often restricted to faecal samples, since *in vivo* investigations with animals and human trials have various drawbacks, such as high costs, ethical constraints, inter-individual variations and limitations in sampling from the small and large bowel (Venema & Van Den Abbeele, 2013; Verhoeckx et al., 2015). Nonetheless, even if they have limitations based on the absence of a physiological host environment, *in vitro* models are reproducible, since they allow better control of the experimental variables than animal or human studies. In general, they are rapid and simple methods and, therefore, relatively inexpensive and cost-effective. Furthermore, they allow a reduction of the samples size when this is a limiting factor (Verhoeckx et al., 2015).

Therefore, several *in vitro* models have been developed to simulate the multistage processes of human gastrointestinal digestion (Alminger et al., 2014; Cascone et al., 2016; Hur, Lim, Decker, & McClements, 2011; Marzorati et al., 2011; Verhoeckx et al., 2015). Among all these models, complex multi-compartmental continuous systems overcome the limitations present on static models, which do not reproduce the dynamic environment of the GIT (*e.g.* pH changes, peristaltic movements, gastric emptying, continuous changes, and secretion flow rates) (Ouwehand & Vaughan, 2006). Nowadays, dynamic gastrointestinal digestion simulators are still limited. The SIMulator Gastro-Intestinal (simgi®, Madrid, Spain) (Barroso, Cueva, Peláez, Martínez-Cuesta, & Requena, 2015) comprises five different compartments system, which simulates the different regions of the GIT such as, stomach (ST), small intestine (SI) and three compartments simulating the ascending (AC), transverse (TC) and descending (DC) regions of the human colon. The simgi® represents a fully computer controlled multi-compartmental system, which allows joint or separated simulation of the gastric and colonic fermentative processes. Thus, this is a flexible modulating system that combines a gastric compartment that simulates peristaltic mixing movements, a reactor simulating the small intestine and three-stage continuous reactors for reproducing the colon region-specific microbiota and its metabolism (Barroso et al., 2015).

Therefore, the aim of the present study was to examine the *in vitro* gastrointestinal digestion of a commercial citrus pectin using the Dynamic Gastrointestinal Simulator (simgi®), and its impact on the subsequent fermentation by the colonic microbiota.

2. Materials and methods

2.1. Samples of pectin

Commercial citrus pectin (trade name Ceampectin®, ESS-4400) was kindly provided by CEAMSA (Porriño, Pontevedra, Spain). Galacturonic acid (GalA) content, degree of methoxylation (DM), molecular weight (Mw) and neutral sugar content of the tested pectin were described in a previous study in our laboratory (Muñoz-Labrador, Moreno, Villamiel, & Montilla, 2018) (Table S1, Supplementary material).

2.2. simgi® model assays digestion

The dynamic gastrointestinal simulator simgi® was used in the operating mode to work with the five units simulating the stomach (ST), small intestine (SI) and the ascending (AC), transverse (TC) and descending colon (DC) regions (Barroso et al., 2015). Fig. 1 shows the experimental protocol of the simgi® trial. The operation of the dynamic model was validated and optimized in previous studies (Barroso et al., 2015; Barroso et al., 2016; Cueva et al., 2015). Faecal slurry was obtained from a healthy volunteer who had no received any antibiotic treatment in the previous 3 months of the experiment. Then, faecal samples were diluted (20% w/w) in sterilised phosphate-buffered saline (PBS; 0.1 mol/L, pH 7.0, Oxoid, Basingstoke, UK) containing 1 g/L sodium thioglycolate as reduced agent. The nutritive medium was adapted from the studies mentioned before and it was constituted by potato starch (Difco™, BD) (7 g/L), glucose (Difco™, BD) (0.4 g/L), yeast extract (Oxoid, ThermoFisher Scientific) (3 g/L), special peptone (Oxoid, ThermoFisher Scientific) (1 g/L), mucin from porcine stomach (Sigma-Aldrich, Merk) (4 g/L) and L-cysteine (Panreac AppliChem) (0.5 g/L). All compounds were dissolved in 1 L of distilled water and sterilized at 121 °C for 21 min with a final pH of 6.0.

The ascending, transverse and descending colon compartments were filled and pre-conditioned with the nutritive medium that feed the system during the stabilization period; 250 mL (AC), 400 mL (TC) and 300 mL (DC) of nutritive medium were added and later inoculated with 20 mL of fresh faecal slurry (20% w/v).

A stabilisation period of 12 days was applied to allow the intestinal microbiota to adapt to environmental conditions present in the colon compartments and to form a stable microbial community (Barroso et al., 2015). This stabilisation was approached by feeding the small intestine with nutritive medium (75 mL, pH 2) mixed with pancreatic juice (40 mL of a solution of 12 g/L $NaHCO_3$ (VWR Chemicals), 6 g/L oxgall dehydrated fresh bile (Difco™, BD) and 0.9 g/L porcine pancreatin (Sigma-aldrich) three times a day during 12 days (Van Den Abbeele et al., 2010). After stabilisation period of the colonic microbiota, the simgi® was subjected to a 2-week experiment, which consisted of adding 240 mL of the commercial citrus pectin per day (3 doses of 80 mL) dissolved in the feeding nutritive medium (30 g/L, pH 3.1). This sample was added directly to the stomach during 14 days, where it was mixed with gastric electrolytes and pepsin by the simulated peristaltic moves, controlling the decrease of pH by adding 0.5 M HCl. After stomach digestion, stomach content was automatically transferred to the small intestine vessel where digestion was performed during 2 h at 37 °C (pH = 7.0). Then, this content was transferred to the following compartment (AC) at a flow rate of 5 mL/min, which simultaneously

activated the transit of colonic content between the AC, TC and DC compartments at the same flow rate. The temperature (37 °C), continuous flushing of nitrogen and pH were continuously controlled by the system. pH in the colonic units was controlled by addition of 0.5 M NaOH and 0.5 M HCl to keep values of 5.6 ± 0.2 in the AC, 6.3 ± 0.2 in the TC and 6.8 ± 0.2 in the DC. Finally, a 1-week washout period was included at the end of the experiment by feeding the simgi® daily with nutritive medium. During the whole study, samples were collected every day at regular time points from the three colon vessels: During stabilisation period (< Day 13, and immediately prior to pectin feeding (Day 13*), during pectin feeding period, samples were also taken in stomach and small intestine compartments (Day 13–27) and after the beginning of washout period (Day 27–34). Finally, all collected samples were immediately centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatants were aliquoted and stored at – 20 °C until being analysed for short-chain fatty acids (SCFA), ammonium and Mw estimation of the tested pectin. Microbial plate count analyses were performed at the time of the sample collection. Pellets were stored at – 80 °C until further analysis of total bacteria and main bacterial groups by qPCR.

2.3. Estimation of the molecular weight (Mw) of pectin

The estimation and monitoring of Mw of pectin samples during the gastrointestinal digestion with the simgi® was carried out by Size Exclusion Chromatography (SEC), according to the method described by Muñoz-Almagro, Rico-Rodriguez, Villamiel, and Montilla, (2018). Analysis was carried out on a LC Agilent Technologies 1220 Infinity LC System 1260 (Agilent Technologies, Boeblingen, Germain), equipped with two consecutive TSK-GEL columns (G5000 PW_{XL}. 7.8 x 300 mm, particle size 10 µm, G2500 PW_{XL}, 7.8 x 300 mm, particle size 6 µm; Tosoh Bioscience, Stuttgart, Germany). Centrifuged samples from the different compartments were first diluted before HPLC analysis: 1/10, 1/4 and 1/2 in HPLC water for ST, SI and AC, TC, DC compartments, respectively. Diluted samples were filtered and eluted (20 µL) with 0.1 M $NH_4CH_3CO_2$ at a flow rate of 0.5 mL/min for 80 min at 30 °C. The eluent was monitored with an Evaporative Light Scattering Detector (ELSD) (Boeblingen, Germain) at 30 °C. Pullulans of Mw 805, 200, 10, 3 and 0.3 kDa were used as standards to calibration. All Mw values specified were weight-average.

2.4. Short-chain fatty acids (SCFA) analysis

SCFA analysis was performed by liquid chromatography using a UV-975 detector following the method described by Sanz et al. (2005). Briefly, samples from the different colon compartments (AC, TC and DC) were filtered and injected on a HPLC system (Agilent Technologies, Germany) equipped with a UV-975 detector and automatic injector. SCFA were separated using a Rezex ROA Organic Acids column (300 x 7.8 mm) (Phenomenex, Macclesfield, UK) thermostated at 50 °C. Mobile phase was sulphuric acid 0.005 mM in HPLC grade water at a flow rate of 0.5 mL/min under isocratic elution. The elution profile was monitored at 210 nm and peaks were compared to standards to be identified. Data acquisition and integration were done using Agilent ChemStation software (Wilmington, DE, USA). Calibration curves of all SCFA were obtained from the analysis of standard solutions of lactic, formic, acetic, propionic, butyric, valeric and isovaleric acid, ranging the concentrations of 1–100 mM.

2.5. Ammonium determination

Ammonium levels were determined using the Ammonium test (Spectroquant Ammonium Test, Merck), following the manufacturer's instructions. Briefly, serial dilutions of an ammonium standard solution (10 g/L) were used to prepare calibration curves. simgi® samples were diluted with deionized water (1:10). Just prior to performing the measurement at 25 °C, 5 mL of reactive NH4-1 and reactive NH4-2 were added to the diluted standards or samples. The mixture was shaken between each reagent addition. Then, the absorbance was quantified at 690 nm. Analyses were performed in duplicate. The results were expressed as mg of NH_4^+ contained in each colon compartment.

2.6. Microbial analyses

2.6.1. Plate counts

Collected samples from the different colon compartments were diluted (1/10) in a physiological solution (0.9%) and were plated on eight types of genera and selective media as follows: Tryptic Soy Agar (TSA) (Becton and Dickson & Company, BD) for total aerobes; Wilkins-Chalgren agar (BD) for total anaerobes; MacConkey agar (BD) for *Enterobacteriaceae;* Enterococcus agar (BD) for *Enterococcus* spp.; MRS agar (Pronadisa) for lactic acid bacteria and Tryptose Sulfite Cycloserine agar (TSC) (Pronadisa) for *Clostridium* spp. Plates were incubated at 37 °C for 24–48 h in an anaerobic chamber (BACTRON Anaerobic/Environmental Chamber, SHELLAB, USA), except for TSA which was incubated in aerobic conditions (Nüve Incubator EN 120, NÜVE, Turkey).

2.6.2. Bacterial DNA extraction and quantitative polymerase chain reaction (qPCR)

Bacterial DNA extraction of pellets from AC, TC and DC compartments was performed using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's recommended protocol. Extracted DNA of all samples was stored at -80 °C until analysis.

The amplification and detection of bacterial DNA was carried out on a ViiA7 Real-Time PCR System (Applied Biosystems). Specific 16 s rRNA-targeting primers were used in this study to determine total bacteria, *Bacteroides* spp, *Bifidobacterium* spp, *Enterobacteriaceae, Enterococcaceae, Lactobacillus* spp, *Faecalibacterium prausnitzii* and the *Clostridium cluster XIVa.* Reactions were done in triplicate in 384-well plates using SYBR® Green PCR Master Mix (Applied Biosystems). Final volume of each amplification reaction was 10 µL: 5 µL of SYBR® Green, 0.3 µL of each primer (10 µM), 3.4 µL of nuclease-free water purified for PCR (Sigma-Aldrich) and 1 µL of DNA template. Thermal cycling consisted of an initial cycle of 95 °C, 3 min, followed by 40 cycles of 95 °C, 15 s and 1 min at the appropriate primer-pair temperature (Gil-Sánchez et al., 2017). In order to quantify bacterial groups, DNA isolated from selected bacterial strains was used, *Bacteroides fragilis* for *Bacteroides, Bifidobacterium longum* for *Bifidobacterium, Blautia coccoides* for *Clostridia XIVa*, *F. prausnitzi* for *Faecalobacterium prausnitzi, Escherichia coli* for *Enterobacteriaceae, Enterococcus falcium* for *Enterococcaceae, Lactobacillus plantarum* for *Lactobacillus* and *B. fragilis* for *total bacteria*. Standard curves were generated by plotting threshold cycles (CT) *vs*. bacterial quantity expressed as colony-forming units (CFU)/mL.

2.7. Statistical analysis

Statistical analysis was performed using SPSS for Windows, version 23.0. One-way analysis of variance (ANOVA) and Tukey's post hoc test was used to determine significant differences among the bacterial group populations obtained after the qPCR analysis and organic acid concentrations to test the main effects of factors studied (time, pectin feeding, compartment). Differences were considered significant at $p < 0.05$ ($n = 3$).

3. Results

Characterisation results showed GalA as the main component with 66.5 ± 0.2% of the total carbohydrates; galactose was the second main component with 20.2 ± 0.1% and rhamnose and arabinose were present with 5.8 and 3.5%, respectively. Glucose, mannose and xylose were also determined in minor values, 1.8, 1.4 and 0.9%, respectively. Interval of Mw and DM of pectin were determined as 100–800 kDa

Table 1
Effect of the gastrointestinal digestion (simgi®) on the estimation and distribution of Mw (Average Mw) of the studied pectin (3%, w/v).

		Concentration of carbohydrate fraction (%)		
Sample/Day	Compartment	350 ± 30 kDa	40 ± 5 kDa	< 18 kDa
Medium	–	–	6.0 ± 1.5	94.0 ± 0.0
Medium + Pectin	–	53.6 ± 1.2	–	46.4 ± 0.0
Day 13*	ST	–	5.7 ± 0.5	94.2 ± 0.1
	SI	–	6.9 ± 0.1	93.0 ± 0.3
	AC	–	5.0 ± 0.2	94.9 ± 0.2
	TC	–	4.2 ± 0.1	95.8 ± 0.1
	DC	–	4.3 ± 0.0	95.5 ± 0.0
Day 15	ST	56.6 ± 0.6	–	42.8 ± 0.9
	SI	52.9 ± 0.4	–	46.7 ± 0.5
	AC	8.5 ± 0.6	15.1 ± 0.8	75.8 ± 0.6
	TC	9.5 ± 0.2	4.8 ± 0.1	85.2 ± 0.3
	DC	9.8 ± 0.1	4.6 ± 0.1	85.1 ± 0.1
Day 24	ST	56.5 ± 1.5	–	42.0 ± 0.3
	SI	47.6 ± 0.3	–	55.9 ± 0.7
	AC	5.4 ± 0.1	17.2 ± 0.9	76.9 ± 0.1
	TC	4.8 ± 0.4	3.5 ± 0.0	91.4 ± 0.5
	DC	4.7 ± 0.2	2.2 ± 0.0	92.8 ± 0.3
Day 27	ST	55.8 ± 0.9	–	43.5 ± 0.2
	SI	47.7 ± 0.8	–	55.7 ± 0.6
	AC	1.9 ± 0.0	17.7 ± 0.6	79.7 ± 0.1
	TC	0.6 ± 0.0	3.7 ± 0.1	95.5 ± 0.1
	DC	0.5 ± 0.0	2.5 ± 0.0	96.8 ± 0.0
Day 34	ST	9.8 ± 0.0	–	89.7 ± 0.4
	SI	7.0 ± 0.1	–	92.8 ± 0.7
	AC	4.0 ± 0.0	–	95.8 ± 0.6
	TC	3.9 ± 0.0	–	96.0 ± 0.2
	DC	2.0 ± 0.0	–	98.0 ± 0.0

Data are expressed as the mean ± SD (n = 2).
*Sample taken before feeding with citrus pectin.

(average 350 ± 30 kDa) and 70.7%, respectively (Ferreira-Lazarte et al., 2018).

3.1. Effect of digestion and fermentation on pectin molecular weight

The behaviour of pectin during the chronic feeding period (Fig. 1) was evaluated by monitoring the Mw during its passage through the different compartments (ST, SI, AC, TC and DC). Table 1 shows the quantitative results obtained by SEC-ELSD determination. Analyses were carried out just before starting the feeding period at day 13 (representing the nutritive medium), and three days during the chronic feeding (Day 15, 24 and 27) as well as the last day of the washout period (Day 34). Results showed a high average Mw for citrus pectin (350 ± 30 kDa) which represented almost 54% of total content when mixed with nutritive medium, whereas the latter was mainly constituted of low Mw carbohydrates (< 18 kDa). Values at Day 13* (before feeding with citrus pectin) showed almost no changes between all compartments. During the chronic feeding, pectin showed no changes in the stomach compartment when compared to the intact pectin (before feeding the system), whereas a slight decrease can be observed after the small intestine passage, showing a high resistance of citrus pectin to the upper gastrointestinal digestion. However, fermentation in the three

different sections of colon gave rise to a remarkable effect on pectin Mw. At this stage, it can be seen the presence of a new chromatographic peak of lower Mw (40 ± 5 kDa) than the peak corresponding to the intact pectin (350 ± 30 kDa), as well as an increase in the abundance of the peak including low Mw carbohydrates (< 18 kDa), probably due to the fermentation of pectin (Fig. 2). Lastly, washout period showed almost no presence of any carbohydrates since feeding with nutritive medium/pectin was substituted with only nutritive medium (Table 1).

3.2. Evolution of the microbial community

The computer-controlled multicompartmental dynamic gastrointestinal model used in this study, allowed us to monitoring the gastrointestinal digestion and fermentation in the different compartments/sections due to its capability to simulate *in vitro* the microbial conditions that characterize the different regions of the gut.

3.2.1. qPCR analysis

Given that important modifications in the HPLC profiles of pectin were found together with slight trends observed by the plate counts (Table 2S, Supplementary material), a qPCR analysis was done at the last day of each period in the AC, TC and DC compartments in order to better assess changes in the microbial population during the *in vitro* fermentation of citrus pectin (Table 2). In general, higher amounts of bacteria were obtained with qPCR as compared with plate counts, which is in consonance with the fact that only a small fraction of the range of gut bacterial groups found had been, up to now, cultured (Zoetendal, Vaughan, & De Vos, 2006). The rest of bacteria were often labelled as “unculturable” due to the generally fastidious anaerobic growth requirements (Allen-Vercoe, 2013). Counts oftotal bacteria group were about 9.5 log copy number/mL at the end of stabilisation period and increased significantly ($p < 0.05$) after citrus pectin feeding in AC and TC whereas in DC a lower and non-significant increase ($p > 0.05$) was determined. In addition, a decrease was observed in all compartments after washout period.

Among all bacterial groups that were studied, a huge and significant increase in counts of *Bifidobacterium* spp, *Bacteroides* spp, *F. prausnitzii* and *Enterobacteriaceae* was observed after the feeding with the citrus pectin in all colon sections with the exception of *Enterobacteriaceae* in TC. Furthermore, an overall and statistically significant decrease was found after the washout period, with some exceptions (*i.e.*, *Bacteroides* spp in AC, or *Enterobacteriaceae* in all colon sections). In contrast, a decrease in *Lactobacillus* spp (in all colon sections) and *Enterococaceae*

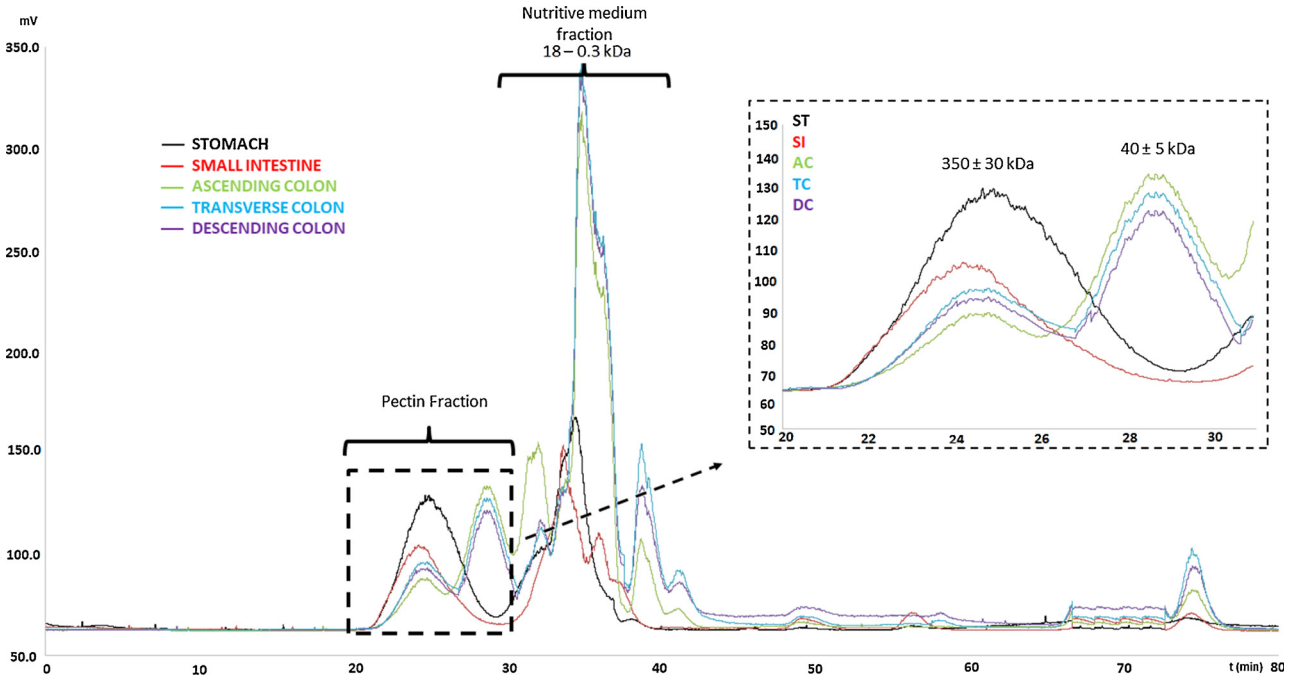

Fig. 2. Qualitative evolution of citrus pectin (Mw distribution) in the five compartments (Stomach (dilution 1/10), Small Intestine (1/4), Ascending Colon (1/2), Transverse Colon (1/2) and Descending Colon (1/2)) during feeding of the dynamic simulator of the gastrointestinal tract (simgi®).

(in TC) was also observed after the feeding period with pectin. These values increased during the washout period for *Enterococcaceae* whereas did not present any changes in *Lactobacillus* spp.

Finally, it should be pointed out that *Clostridia XIVa* group was also determined showing no changes in all compartments between stabilisation, chronic and washout period (data not shown).

3.3. Metabolic activity

The metabolic activity of the microbiota before, during and after feeding with pectin in the different colonic reactors of the simgi® was evaluated by monitoring the content of SCFA (fermentative metabolism) and of ammonium (proteolytic metabolism).

Table 2
Mean values (n = 3) of the qPCR data as copy number/mL for the microbial groups analysed in the ascending (AC), transverse (TC) and descending colon (DC) of the dynamic gastrointestinal model (simgi®) at the end of stabilization period (day 13), chronic intake (day 27) and washout period (day 34) with citrus pectin. Values in brackets represents the data as the log10 of copy number/mL.

Bacteria group	Compartment	Stabilisation period Day 13*	Chronic intake period Day 27	Washout period Day 34
Total bacteria	AC	3.1×10^9 (9.49 ± 0.05)^a	6.6×10^9 (9.82 ± 0.02)^c	4.8×10^9 (9.68 ± 0.04)^b
	TC	3.4×10^9 (9.50 ± 0.23)^a	1.5×10^{10} (10.16 ± 0.01)^b	3.0×10^9 (9.48 ± 0.06)^a
	DC	2.7×10^9 (9.43 ± 0.01)^a	3.9×10^9 (9.59 ± 0.02)^a	1.6×10^9 (9.14 ± 0.33)^a
Lactobacillus	AC	1.8×10^5 (5.24 ± 0.13)^b	$\leq 10^4$ ($\leq$ 4)^a	$\leq 10^4$ ($\leq$ 4)^a
	TC	6.7×10^5 (5.79 ± 0.24)^b	$\leq 10^4$ ($\leq$ 4)^a	$\leq 10^4$ ($\leq$ 4)^a
	DC	3.6×10^5 (5.53 ± 0.18)^b	2.8×10^4 (4.39 ± 0.28)^a	1.5×10^4 (4.03 ± 0.48)^a
Bifidobacterium	AC	2.8×10^5 (5.44 ± 0.04)^a	3.7×10^8 (8.56 ± 0.06)^c	2.8×10^6 (6.43 ± 0.13)^b
	TC	7.6×10^5 (5.87 ± 0.14)^a	1.1×10^8 (8.02 ± 0.09)^c	6.0×10^6 (6.77 ± 0.05)^b
	DC	3.2×10^5 (5.50 ± 0.07)^a	3.4×10^8 (8.54 ± 0.02)^c	9.6×10^6 (6.98 ± 0.02)^b
Bacteroides	AC	2.8×10^8 (8.45 ± 0.08)^a	1.7×10^9 (9.24 ± 0.01)^b	1.5×10^9 (9.18 ± 0.02)^b
	TC	7.0×10^8 (8.85 ± 0.01)^a	4.4×10^9 (9.64 ± 0.03)^b	6.2×10^8 (8.79 ± 0.07)^a
	DC	3.4×10^8 (8.53 ± 0.05)^a	8.5×10^9 (8.93 ± 0.02)^c	5.0×10^8 (8.70 ± 0.02)^b
Faecalobacterium prausnitzii	AC	$\leq 10^5$ ($\leq$ 5)^a	2.8×10^7 (7.43 ± 0.14)^b	$\leq 10^5$ ($\leq$ 5)^a
	TC	$\leq 10^5$ ($\leq$ 5)^a	1.3×10^8 (8.08 ± 0.18)^b	$\leq 10^5$ ($\leq$ 5)^a
	DC	$\leq 10^5$ ($\leq$ 5)^a	1.6×10^7 (7.17 ± 0.21)^b	1.1×10^5 (5.05 ± 0.10)^a
Enterococcaceae	AC	$\leq 10^4$ ($\leq$ 4)^a	4.9×10^5 (5.68 0.06)^b	$\leq 10^4$ ($\leq$ 4)^a
	TC	6.8×10^6 (6.83 ± 0.05)^b	1.8×10^4 (4.19 0.28)^a	2.5×10^6 (6.40 0.02)^b
	DC	1.1×10^6 (6.05 ± 0.04)^a	3.2×10^5 (5.50 0.03)^a	6.4×10^5 (5.69 0.45)^a
Enterobacteriaceae	AC	9.5×10^6 (6.98 ± 0.02)^a	4.6×10^8 (8.66 ± 0.03)^b	3.6×10^8 (8.55 ± 0.07)^b
	TC	2.1×10^8 (8.32 ± 0.08)^{ab}	1.7×10^8 (8.21 ± 0.19)^a	4.1×10^8 (8.60 ± 0.07)^b
	DC	5.6×10^7 (7.70 ± 0.24)^a	1.9×10^8 (8.23 ± 0.25)^b	2.3×10^8 (8.37 ± 0.02)^b

^{a,b,c} Significant differences ($p < 0.05$, ANOVA) were determined for $\log_{10}$ values (in brackets) for the same bacterial group. Letters represent significant differences between days for the same compartment in each bacterial group.
*Sample taken before feeding with citrus pectin.
Standard deviation values are in brackets.

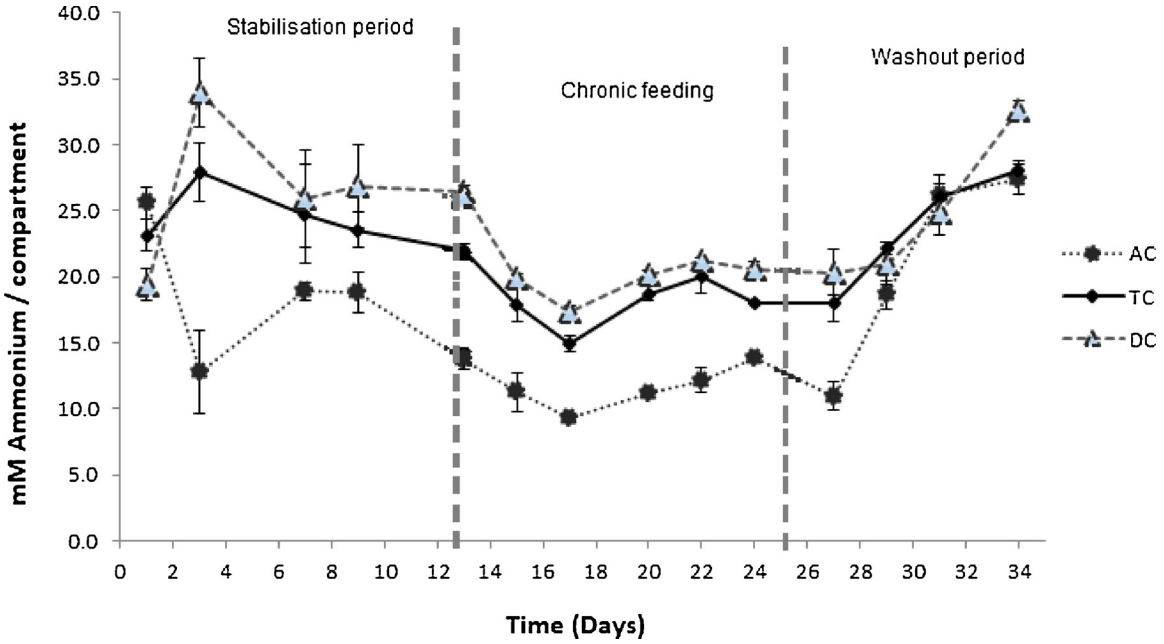

Fig. 3. Ammonium evolution (mM) during gastrointestinal digestion of citrus pectin in the simgi®.

3.3.1. Ammonium determination

Evolution of ammonia during the simulation of the gastrointestinal digestion in the three colon compartments is shown in Fig. 3. A slight but significant decrease ($p < 0.05$) in ammonium concentration is observed during the feeding with the citrus pectin, as compared with stabilisation and washout periods. Also, as it has been observed in previous studies with the same system (simgi®) (Barroso et al., 2015; Barroso et al., 2016; Gil-Sánchez et al., 2017), ammonium concentration gradually increased from AC to the DC compartment because of the accumulation of products in the system, which lacks of any absorption steps between the different compartments. These values showed that proteolytic metabolism occurred through the entire colon compartments during the whole experiment, but it was substantially diminished during the chronic feeding period with citrus pectin.

3.3.2. Short-chain fatty acid (SCFA) analysis

The major end-products of indigestible carbohydrates metabolism by the colonic microbiota are SCFA. SCFA evolution (mM) during the stabilisation period, chronic feeding of citrus pectin and washout period is shown in Fig. 4. SCFA concentrations presented no changes during the stabilisation period in all three compartments. During the chronic feeding with pectin these levels showed a significant increase in all major SCFA (acetate, propionate and butyrate) which decreased after elimination of nutritive medium/pectin administration. As expected, SCFA production consisted mainly of acetate, butyrate and propionate with small amounts of lactate and valerate in all compartments. Acetate was the most abundant SCFA, followed by butyric and propionic acid, showing increases up to 297, 92 and 60%, respectively, after chronic feeding, as compared to the initial levels (Day 13). Afterwards, they showed a

considerable decrease during washout period started. Overall, the total SCFA average molar production was compartment-dependent, being higher in the transverse and descending colon. Regarding minor SCFA, results also showed significant values of valerate in all three compartments (Khodaei, Fernandez, Fliss, & Karboune, 2016). Formic acid was detected in the AC compartment reaching values of 2.7–8.0 mM whereas TC and DC presented concentrations below 1.1 mM. Lactic acid production was only detected at fermentation days with the citrus pectin (1.3, 8.7 and 4.0 mM in ascending, transverse and descending colon, respectively). Although lactate is not a SCFA, it is usually considered in the metabolism of bacteria as a product of saccharolytic fermentation. Furthermore, valerate has been described as a primary end product of lactate fermentation (Almeida et al., 2017; Unger et al., 2016; Yoshikawa et al., 2018).

4. Discussion

Several studies have shown that oligosaccharides deriving from pectins exert bifidogenic activities. Furthermore, there are also studies that have demonstrated a significant growth of bacteria in intact pectins suggesting a potential role of this polysaccharide as a prebiotic (Ferreira-Lazarte et al., 2018; Gómez, Gullón, Yáñez, Schols, & Alonso, 2016; Yang, Martínez, Walter, Keshavarzian, & Rose, 2013). In fact, in a static *in vitro* study, we have recently shown that either pectin or enzymatically-modified pectin from different by-products stimulates beneficial bacteria of colonic microbiota (Ferreira-Lazarte et al., 2018). However, no investigation has been carried out on the properties of pectin as a substrate for fermentation including a previous passage through the upper gastrointestinal tract. This study remarks, for the first time, the use of the simgi® to evaluate the effect of the upper gastrointestinal digestion on a commercial citrus pectin and its effect on colonic microbiota metabolism.

Pectin taken from the stomach compartment showed almost no changes when compared to the initial status, whereas samples taken from the small intestine revealed some loss of pectin, 6.5, 15.7 and 14.7% for Day 15, Day 24 and Day 27, respectively. Holloway et al. (1983) observed a loss of pectin of 15–40 % in an *in vivo* study with ileostomy samples. In the same way, Saito et al. (2005) found that approximately 90% of ingested pectin was recovered in the terminal ileum in an *in vivo* study collecting endoscopy retrograde samples. These studies attributed the loss of pectin to the possible degradation by bacteria within the digestive tract, especially the terminal ileum. However, given that in our prototype of digestion the presence of bacteria is confined to the colon compartments, changes observed in the Mw of pectin after its passage through the SI could be related to other chemical effects due to the interaction with pancreatic fluids and bile salts (Miller, Buchanan, Eastwood, & Fry, 1995).

Regarding the effect of digested pectin on microbiota, results obtained showed that citrus pectin favourably impacts on microbiota composition and functionality in the three compartments (AC, TC and

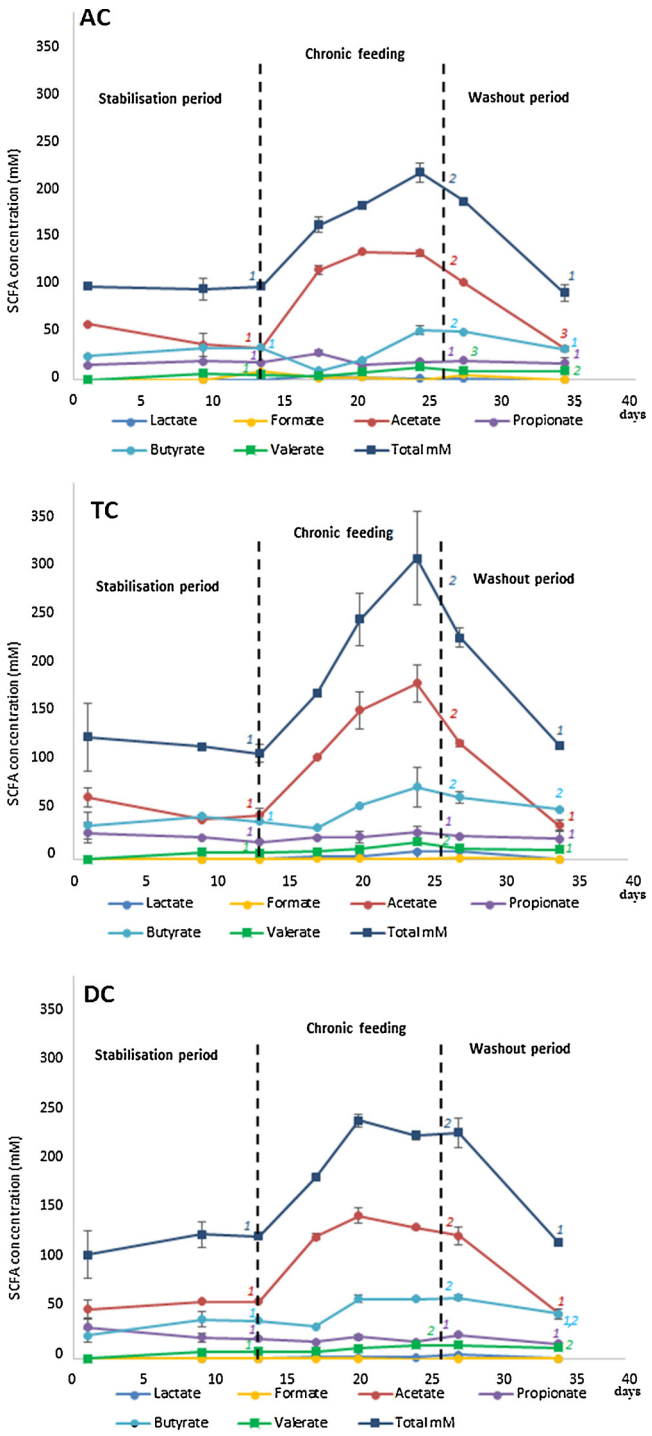

Fig. 4. Evolution in concentration of SCFA in the ascending (AC), transverse (TC) and descending colon (DC) of the simgi® during the stabilisation (D1-D13), feeding (D13-D27) and washout period (D27-34) with citrus pectin solution (3 g/L). ^{1,2,3} Numbers represent differences ($p < 0.05$) between the data at the end of each period.

DC) of the simgi® model.

Pectin fermentation produced an increase in the counts of total bacteria, compared to the initial state, with significant increases in the proximal regions (ascending and transverse colon) probably due to the content of polysaccharide coming from the small intestine. Furthermore, high methoxyl pectins, as it is the case of citrus pectin here assayed, have showed a slower fermentation in the large intestine of rats, which allows the fermentation to take part in all three compartments (Dongowski et al., 2002).

According to some authors, increments up to 0.5–1.0 $\log_{10}$ in *Bifidobacterium* populations could be considered as a major shift in the gut microbiota towards a potentially healthier composition of intestinal microbiota (Kolida & Gibson, 2007). *Bifidobacterium* and lactobacilli have been traditionally considered as the major microbial targets for prebiotic action, due to their beneficial effects (Roberfroid et al., 2010). Similar values of *Lactobacillus* spp and *Bifidobacterium* spp populations at the end of stabilisation period were observed (Table 2). Increases up to 2.15–3.12 $\log_{10}$ in *Bifidobacterium* group was determined in all compartments, being the highest increase of all bacteria determined. This could be attributed to the high galactose/arabinose content of the studied pectin (23.8%) (Di et al., 2017; Onumpai et al., 2011). However, unlike *Bifidobacterium, Lactobacillus* group showed a significant decrease after feeding with citrus pectin. In related studies, Olano-Martin, Gibson, & Rastall, (2002) reported that both POS and citrus pectin significantly increased the number of *Bifidobacteria*, whereas lactobacilli numbers only increased with POS although this increase was not statistically significant. Furthermore, Chen et al. (2013), showed an increase of *Bifidobacteria* during the *in vitro* fermentation of apple pectin (DM 70%) and POS, whereas *Lactobacillus* population presented no changes or even similar values for pectin compared to the negative control after 24 h of fermentation. Li, Zhang, and Yang, (2018) also showed a decrease of *Lactobacillus* when feeding rats with pectin extracted from citrus peels in an *in vivo* study.

Faecalobacterium prausnitzii values reported to be the second highest increase during the fermentation of pectin in the simulator (2.17–3.03 $\log_{10}$). *F. prausnitzii* is one of the most abundant commensal bacteria in the healthy large intestine and is one of the main producers of butyrate in the human colon (Louis, Scott, Duncan, & Flint, 2007; Louis, Hold, & Flint, 2014). Furthermore, low *F. prausnitzii* levels were correlated with the recurrence of inflammatory bowel disease and it has confirmed to have anti-inflammatory effects (Onumpai et al., 2011; Sokol et al., 2008). Likewise, it has been suggested that this bacterium could be a good probiotic candidate to counterbalance dysbiosis in Crohn's disease patients (Scott, Martin, Duncan, & Flint, 2014; Sokol et al., 2009). Moreover, previous studies have shown that this bacterium could have a major role in pectin utilization in comparison with other two abundant pectin-utilizing species, *Bacteroides thetaiotaomicron* and *Eubacterium eligens* (Lopez-Siles et al., 2012).

Bacteroides population showed also a high increase being the third highest increase of all bacteria determined with values of 0.4 – 0.8 $\log_{10}$. *Bacteroides* are one of the enterotypes of the human microbiota, which are responsible for the major part of polysaccharide digestion occurring in the human large intestine (Flint, Scott, Duncan, Louis, & Forano, 2012; Salazar et al., 2009). In fact, many strains from human faeces can produce various pectinolytic enzymes, including polygalacturonase, pectin methylesterase, extracellular and cell-associated pectate lyase (Dekker & Palmer, 1981; Jensen & Canale-parola, 1986). Hence, *Bacteroides* could be involved in cross-feeding with *Bifidobacteria* by releasing breakdown products of pectin which might be utilized by the latter.

Enterococaceae and *Enterobacteriaceae* groups presented different behaviour compared to the bacteria mentioned before. Significant increase were found in the AC for both bacteria during the feeding period with citrus pectin, whilst TC showed a decrease of *Enterococaceae* and no significant change for *Enterobacteriaceae*. Nevertheless, a significant increase in *Enterobacteriaceae* population was found in DC whereas stable levels were observed for *Enterococaceae* after feeding with citrus pectin.

Concerning the proteolytic and saccharolytic activity of microorganisms, SCFA concentrations increased during the chronic feeding with pectin. Lactic and formic acid were observed in low concentrations since produced lactic acid is considered to be an intermediate metabolite and can be further metabolized within the colon and turned into butyric and propionic acids through cross-feeding by gut bacteria

(Duncan, Louis, & Flint, 2004; Reichardt et al., 2014). Similarly, formic acid is used by microorganisms that have a particularly important role in anaerobic metabolism, *via* interspecies cross-feeding interactions (Louis et al., 2014). Results obtained showed an increase for valerate during the pectin fermentation. Khodaei et al. (2016) also reported a small amount of valerate when testing a galactose/rhamnose rich polysaccharide with similar values compared with recognised prebiotic, such as FOS.

The major end-products of saccharolytic fermentation are acetate, propionate and butyrate, which have a combined concentration of 50–150 mM in the colon (Louis et al., 2014). High levels of SCFA are desirable since, among other benefits, the corresponding decrease in the pH values can suppress the growing of pathogenic bacteria. Fig. 4 shows a significant high increase of these compounds, being acetate the major SCFA produced followed by butyric and propionic, respectively.

Given the complexity of the human microbiota, it is challenging to attribute a particular fermentation end-product to a specific bacterial group, however, acetate is typically generated *via bifidus* pathway, and more specifically it is a major end-product of *Bifidobacterium* fermentation (Sanz et al., 2005). Thus, the high production of acetate observed in our study can be ascribed to the growth of *Bifidobacterium* population in presence of pectin. The high increase of propionate concentrations after feeding the system with pectin is in good agreement with the increase in *Bacteroides* population, one of the main propionate-producing bacteria in the human colon. Propionate has also been shown to exert beneficial effects such as protective role against carcinogenesis through the decrease in human colon cancer cell growth (Jan et al., 2002; Hinnebusch, Meng, Wu, Archer, & Hodin, 2002). In addition, propionate and formate were reported to reduce the activity of *E. coli* and *Salmonella* at pH 5 (Gullón, Gullón, Sanz, Alonso, & Parajó, 2011; Topping & Clifton, 2001). Significant increases in butyrate concentrations were also observed in all three compartments, with the second highest levels after acetate. *F. prausnitzii* might utilise apple, citrus and sugar beet pectin as a source of growth and butyrate formation as shown by using pure cultures and *in vitro* models (Chung et al., 2017; Gómez et al., 2016; Lopez-Siles et al., 2012; Onumpai et al., 2011). Thus, butyrate levels concur with the high increase of *F. prausnitzii* population observed. Furthermore, higher levels of butyrate can also be explained due to cross-feeding between *Bifidobacteria* and *F. prausnitzii* since the latter is able to use the acetate produced by *B. adolescentis* thereby boosting butyrate formation (Rios-Covian, Gueimonde, Duncan, Flint, & De Los Reyes-Gavilan, 2015). Apart from these effects, butyrate is known to affect several components of the colonic defence barrier, resulting in enhanced protection against luminal antigens (Hamer et al., 2008; Havenaar, 2011).

Regarding ammonia concentration, Fig. 3 showed a slight but significant decrease in ammonium concentration during the feeding with citrus pectin. It is noteworthy that lower proteolytic activities are usually associated with health-promoting effects (Ichikawa & Sakata, 1998), since it can be a potential carcinogenic agent at relatively low concentrations, as has been shown by the increase in mucosal damage and colonic adenocarcinoma in a rat model (Louis et al., 2014; Windey, de Preter, & Verbeke, 2012). A significant positive correlation was observed between SCFA levels and ammonia production where more acidic conditions favour the excretion of ammonia due to the protonation and formation of poorly absorbed ammonium ion (Louis et al., 2014).

5. Conclusions

The *in vitro* study using the dynamic gastrointestinal simulator simgi® pointed out the high indigestibility of citrus pectin, since a reduced hydrolysis (~12%) was detected in the upper gastrointestinal tract (ST and SI), being mainly due to chemical interactions with pancreatic fluids and bile salts. Findings also highlight the important role played by pectin in stimulating beneficial bacteria such as *Bifidobacteria, F. prausnitzii* and *Bacteroides* (especially in the first two bacterial groups). A high increase in acetate, propionate and butyrate concentration was observed due to fermentation of pectin by the microbiota but also to cross-feeding interactions between different bacteria. Increase in SCFA also produced a decrease in ammonia concentration, which is associated with health-promoting effects. This is the first study of gastrointestinal digestion and fermentation of pectin in a dynamic gastrointestinal simulator and, although further *in vivo* studies should be conducted, the data obtained confirmed the potential of pectin to be considered shortly as emergent prebiotics with a possible use for human consumption.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2018.11.088.

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