Analysis, structural characterization and bioactivity of oligosaccharides derived from lactose

F. Javier Moreno, Antonia Montilla, Mar Villamiel*, Nieves Corzo and Agustín Olano

Instituto de Investigación en Ciencias de la Alimentación (CIAL) (CSIC-UAM). CEI (UAM+CSIC), Nicolás Cabrera, 9, 28049-Madrid (Spain).

*Corresponding author

Instituto de Investigación en Ciencias de la Alimentación, CIAL (CSIC-UAM),

C/ Nicolás Cabrera 9, Campus de la Universidad Autónoma de Madrid.

E-28049, Madrid, Spain.

Tel +34 910017951

Fax +34 910017905

E-mail: m.villamiel@csic.es

Abbreviations:

DP: Degree of polymerization

FOS: Fructooligosaccharides

GOS: Galacto-oligosaccharides

OsLu: Oligosaccharides derived from lactulose

HMO: Human milk oligosaccharides

SCFA: Short chain fatty acids

TMS: Trimethylsilyl

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ABSTRACT

The increasing interest for prebiotic carbohydrates as functional food ingredients has promoted the synthesis of galacto-oligosaccharides and new lactose derivatives. This review provides a comprehensive overview on the chromatographic analysis, structural characterization and bioactivity studies of lactose-derived oligosaccharides. The most common chromatographic techniques used for the separation and structural characterization of this type of oligosaccharides, including GC and HPLC in different operational modes, coupled to various detectors are discussed. Insights on oligosaccharide MS fragmentation patterns using different ionization sources and mass analyzers, as well as data on structural analysis by NMR spectroscopy are also described. Finally, this article deals with the bioactive effects of galacto-oligosaccharides and oligosaccharides derived from lactulose on the gastrointestinal and immune systems, which support their consumption to provide significant health benefits.

1 Introduction

Traditionally, whey was considered an abundant waste material of the cheese industry with very limited value as animal feed. However, application of whey ultrafiltration in the cheese industry, allowed whey protein concentrates to be recovered and used as ingredients in a variety of food industries because of their numerous functional properties, such as, bulking, foaming, and water binding [1]. Advances in processing technologies have also led to the industrial production of whey protein fractions with different biological activities used as ingredients in various products including infant formulas, specialized enteral and clinical protein supplements and sport nutrition products [2].

As the result of whey protein recovery, large amounts of whey permeate containing mainly lactose are generated. Lactose itself has some functional properties that allow to be used in the food and pharmaceutical industries; however, world lactose demand is less than its availability. To overcome this limitation several processes to obtain lactose derivatives with other functional and bioactive properties were commercially developed. Since lactose is a reducing carbohydrate**,** it can be isomerized, reduced or oxidized to give lactulose (4-O-β-galactopyranosyl-D-fructose), lactitol (4-O-β-galactopyranosyl-D-glucitol) or lactobionic acid (4-O-β-galactopyranosyl-D-gluconic acid), respectively. These products are marketed for years and used in the food and pharmaceutical industries. Other lactose derivatives with reported bioactive properties are galacto-oligosaccharides (GOS), tagatose, lactosucrose or sialyllactose [3, 4].

In the last few years, increasing interest in the consumption of prebiotic carbohydrates has been observed; therefore, the development of new approaches to the

synthesis of carbohydrates with bioactive properties is growing up. Consequently, one of the most appealing uses of whey permeate is the enzymatic synthesis of prebiotic GOS. In this context, the efficient synthesis of a number of lactose-related oligosaccharides, such as oligosaccharides derived from lactulose (OsLu), 2-α-glucosyl-lactose or lactulosucrose, have been recently developed. This article reviews the production, analysis and structural characterization of GOS, as well as that of other lactose-derived oligosaccharides with potential functional properties. Finally, data warranting the bioactive properties of GOS and OsLu are also discussed.

2 Synthesis of oligosaccharides from lactose

Oligosaccharides from lactose can be obtained using chemical and enzymatic methods. The transgalactosidase activity of lactase whereby a wide range of GOS are produced from lactose has been known for over 60 years [5]. However, it has gained renewed interest in the past few years due to the recognition of GOS as prebiotics [6, 7], being the most recent oligosaccharides derived from lactose to become commercially available. The composition of the oligosaccharide mixture obtained during transgalactosylation of lactose is highly affected by several factors including the enzyme source, lactose concentration, substrate composition, and reaction conditions (temperature, time and pH) [4]. In transgalactosylation reactions using lactose as a single substrate, the galactose released during enzymatic hydrolysis of lactose is transferred to another lactose molecule, being linked to the galactose moiety by β(1→6), β(1→3) or β(1→4) glycosidic bonds, depending on the enzyme source. The trisaccharides formed may be elongated by new linked galactosyl moieties [4]. The galactosyl residue may be transferred to the glucose released to give allolactose.

Quantitatively, allolactose is one of the major oligosaccharides produced by neutral pH β-galactosidases [6].

Although lactulose is a well-known lactose derivative with multiple health benefits [8], its use as substrate for oligosaccharide synthesis has not been undertaken until recently [9]. β-Galactosidase has the ability to hydrolyze lactulose and transfer the galactosyl residue to the galactosyl moiety of another lactulose molecule. As in the case of transgalactosylation of lactose, the released galactose moiety is linked by β(1→6), β(1→3) or β(1→4) glycosidic bonds [10]. When transgalactosylation reactions are carried out in presence of other galactosyl acceptors, a number of different galactosylated oligosaccharides may be originated during lactose hydrolysis [11, 12].

Oligosaccharides derived from lactose can also be obtained via transglycosylation catalyzed by glycoside hydrolases using different glycosyl donors and lactose as acceptor. This method has been used to prepare lactose derivatives as lactosucrose, trisaccharide produced from the transfer of a fructosyl moiety of sucrose to lactose, catalyzed by β-fructofuranosidases or levansucrases [13, 14]. Similarly, 2-α-D-glucopyranosyl-lactose is produced using dextransucrase which transfers glucose from sucrose (donor) to lactose (acceptor) by linking mainly an α(1→2)-glucosyl bond [15].

Since GOS are reducing carbohydrates, they also can be chemically isomerized at their reducing glucose end using basic catalysts such as sodium aluminate, and converted to the corresponding keto-sugar [16].

3 Chromatographic analysis

The synthesis and characterization of GOS formed during enzymatic hydrolysis of lactose has been a subject of research for many years, and a variety of well-established classical methods are now available [17-21]. Although di- and trisaccharides have been well characterized, the chemical structure of higher molecular weight oligosaccharides has not been investigated in detail. Moreover, since transglycosylation in lactose solutions may be performed under a number of different conditions [4, 16, 22, 23], new lactose derivatives are continuously being isolated and characterized.

Among the main analytical techniques currently used in carbohydrate analysis (chromatographic, electrophoretic and spectroscopic), Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) are methods of choice. Despite capillary electrophoresis (CE) enables high-resolution analysis of heterogeneous mixtures of oligosaccharides derived from various sources [24], there are only a few studies dealing with the CE analysis of GOS [25-27]. In these cases, GOS were previously derivatized to be determined by UV or laser-induced fluorescence detection. Similarly, for GC analysis, carbohydrates must first be converted into volatile derivatives whereas in HPLC, samples can, in most cases, be analyzed without prior derivatization. Additionally, the development during the last decades of a wide range of new support materials and/or stationary phases operating under different separation modes have improved the separation of structurally related carbohydrates by HPLC. Therefore, nowadays HPLC, combined with pulsed amperometric, refractive index and fluorescence detectors or Mass Spectrometry (MS), is the most used chromatographic technique for the analysis of oligosaccharides.

3.1 HPLC separation modes

Reverse phase (RP)-HPLC is commonly used for the analysis of carbohydrates [28-30]. The separation in this mode of HPLC is through hydrophobic interactions, the stationary phase is non-polar (e.g. silica-based modified with octadecyl functional group) and the mobile phase is polar (e.g., binary mixture of water and a miscible polar organic solvent such as methanol or acetonitrile). This operating mode explains that oligosaccharide separations are normally difficult due to the polar nature of these compounds; thus, to overcome these issues, oligosaccharides are commonly derivatized with hydrophobic chromophores or fluorophores enabling separation and sensitive detection [31]. RP-HPLC has been successfully applied to the study of oligosaccharides naturally present in milk, mainly focused on oligosaccharides containing *N*-acetyl amino groups that provide a good chromophore for ultraviolet (UV) detection at low level [32] or after derivatization with different reagents [33-35].

The analysis of oligosaccharides formed during chemical or enzymatic modifications of lactose is mainly accomplished by High Performance Anion Exchange Chromatography (HPAEC) using CarboPac® PA columns with sodium hydroxide and sodium acetate solutions as mobile phases. Under these conditions, the weakly acidic nature of carbohydrates gives highly selective separations so that a number of studies on the use of HPAEC in the analysis of lactose-derived oligosaccharides have been performed during the last few years [26, 36-42]. This is a powerful HPLC operating mode capable of separating oligosaccharides based on structural features such as size, charge, composition, anomericity and linkage isomerism [43]. Thus, the larger the oligosaccharide structure, the greater its negative charge, and the later it elutes. This is attributed to the increasing number of ionized hydroxyl groups in the alkaline solution, which produced stronger molecular adsorption onto the anion exchange stationary phase. Apart from charge and size, differences in the oligosaccharides tertiary structure

and structural modifications of oligosaccharides during isolation or release have been also shown to affect oligosaccharide retention on HPAEC columns [44].

Resins loaded with Ca^{2+} have also been used for the analysis of GOS formed by the action of a commercial grade lactase from *Bacillus circulans* [45] and the study of GOS present in dried buttermilk has been successfully performed using a cation exchanger column in Ca^{2+} form with deionized water as mobile phase [46].

Hydrophilic interaction chromatography (HILIC), which involves the use of hydrophilic stationary phases and hydrophobic mobile phase, has been extensively applied for the analysis of *N*- and *O*-glycans and some studies have been performed on the analysis of human milk oligosaccharides (HMO) [33]. Analysis of neutral oligosaccharides is currently achieved using different stationary phases (e.g. silica particles or chemically modified monolithic silica columns.). Recently, satisfactory resolution of complex mixtures of different commercial GOS was achieved on ethylene bridge hybrid amide stationary phase, using acetonitrile:water with 0.1% ammonium hydroxide as mobile phase [47].

Quantification and analysis of OsLu (up to degree of polymerization, DP, of 6) has also been successfully accomplished by liquid chromatography on a graphitized carbon column. The used Hypercarb^{TM} column efficiently resolved the different oligosaccharides on the basis of their DP [48].

With respect to detectors for HPLC analysis of lactose derived oligosaccharides, UV detector can be applicable only to carbohydrates with UV-absorbing chromophores such as sialic acid, aldonic acid or amino residues found in HMO [32], whereas neutral carbohydrates present in milk require prior derivatization [33-35]. Among the detection systems that can be coupled to HPAEC, pulsed amperometric detection (PAD) enables the universal and highly sensitive detection, reportedly in the picomole range [49], of all

carbohydrates [50], being used to analyze mixtures of GOS formed during enzymatic lactose hydrolysis by β-galactosidases from *Lactobacillus reuteri* [26], *L. plantarum* [36], *Kluyveromyces lactis* [37, 38], *B. circulans* [39] and *Aspergillus aculeatus* [40]. HPAEC-PAD has also been used to quantify levels of GOS in commercial lactose-free UHT dairy products [41] and commercial fermented milks [42], as well as to the study of the new oligosaccharides formed during enzymatic hydrolysis of lactulose by β-galactosidase from *K. lactis* [9] and *A. aculeatus* [10]. **Figure 1** shows a HPAEC-PAD carbohydrate profile of the reaction mixture resulting from the enzymatic synthesis of OsLu after 7 h of incubation.

HPLC coupled to a refractive index detector (HPLC-RID) has been successfully applied for the determination of GOS during lactose hydrolysis by β-galactosidases from *A. oryzae* [50, 51] and *B. circulans* [45]. Using β-galactosidases from *B. circulans*, *A. oryzae*, *K. lactis*, and *K. fragilis*, clear differences between the β-galactosidase activities were found concerning amount and size of oligosaccharides produced [52]. HPLC-RID has also been applied to analyze the formation of lactulosucrose by the *Leuconostoc mesenteroides* B-512-F dextransucrase which catalyzes the transfer of the glucosyl residue from sucrose to lactulose [23]. Although this technique has been widely used in the study of lactose derived oligosaccharides, its known low sensitivity has been recently highlighted in a comparative analysis by HPAEC-PAD and HPLC-RID of the oligosaccharide mixture obtained during the enzymatic synthesis of 2-α-D-glucopyranosyl-lactose. The HPAEC-PAD chromatograms showed the presence of a series of peaks which could correspond to trisaccharides or oligosaccharides with higher DP that were not detected by HPLC-RID [53]. Additionally, RID is not appropriate for use with gradient elution. However, RID is considerably less expensive that PAD,

whose higher sensitivity may not always be needed, in particular when analyses are restricted to major carbohydrates [30].

3.2 GC methods

GC has been widely used for the analysis of carbohydrates because is a rapid, simple, relatively cheap, and powerful analytical technique commonly found in academic and industrial laboratories. It is a technique with high resolving power, sensitivity and selectivity which enables higher oligosaccharides determination in foods that are often present at low concentrations. The most widely used GC detector for carbohydrate analysis is the flame ionization detector (FID). However, the coupling of GC to MS detectors has greatly contributed to identification and quantification of carbohydrates, mainly in complex mixtures with oligosaccharides which present equal DP as it will be explained below. This fact together with the development of capillary and high-temperature columns to analyze carbohydrates with a DP of up to eleven makes GC a technique with high potential for oligosaccharides analysis [29, 54-56].

Trimethylsilyl (TMS) oximes are widely used derivatives for GC analysis of many oligosaccharides since they produce only two peaks corresponding to the *syn* (E) and *anti* (*Z*) forms for reducing sugars and only one peak for non-reducing carbohydrates. Cardelle-Cobas et al. [57] employed this derivatization to analyze by GC-FID mono-, di- and trisaccharides formed during lactose transgalactosylation using two commercial β-galactosidase preparations, Pectinex Ultra SP-L and Lactozym 3000 L HP G. Carbohydrates from reaction mixtures were separated, using a fused silica capillary column coated with CP-Sil 5CB (methyl siloxane). This same method was also employed by Montilla et al. [58] to quantify di- and trisaccharide production during transglycosylation of lactose using β-galactosidases from *K. lactis*. Enzymatic reactions

were carried out using different buffers and influence of cations Na^+ and K^+, as well as of anions acetate and phosphate was investigated. The results showed that the formation of these carbohydrates was higher in presence of Na^+ regardless of the anion used.

Corzo-Martínez et al. [22] identified by GC-MS oligosaccharides formed by transgalactosylation of isomerized cheese whey permeate using β-galactosidase from *B. circulans*. TMS oxime derivatives were separated in a HP-5 MS (5% phenyl methyl siloxane) capillary column. The same column and derivatization procedure was successfully used for the analysis of 2-α-D-glucopyranosyl-lactose and leucrose formed during enzymatic hydrolysis of mixtures sucrose:lactose and sucrose:cheese whey permeate using a dextransucrase from *L. mesenteroides* [53]. Alditol acetates derivatives have also been used for sugar GC analysis due to their stability and the simplicity of the resulting chromatograms. Coulier et al. [59] used these derivatives to identify the glycosidic linkages present in the commercial GOS mixture Vivinal® GOS by GC-MS using a DB-225 ms capillary column.

Also, monosaccharide composition (fucose, galactose, glucose and glucosamine) of HMO was determined by GC-FID of alditol acetate derivatives [60]. First, oligosaccharides were hydrolyzed using trifluoroacetic acid, reduced with borohydride and transformed in *O*-acetylated derivatives that were separated isothermally in a DB-225 ms capillary column.

Because oligosaccharides usually appear in complex matrices, purification steps are required before analysis. In those cases where the study is guided to a specific carbohydrate or a group of carbohydrates, fractionation steps are also required to provide an enrichment of the samples [29]. Hernández-Hernández et al. [61] used four fractionation techniques (diafiltration, yeast treatment, activated charcoal adsorption and Size Exclusion Chromatography, SEC) to purify the prebiotic commercial mixture

Vivinal® GOS. TMS oximes of oligosaccharides from treated mixtures were analyzed by GC-MS using a HT5 (5% phenyl polysiloxane-carborane) column. Yeast or activated charcoal (with 1% of ethanol) treatments selectively removed monosaccharides; however, SEC was the most appropriate method to obtain GOS fractions (DP up to 8) with high purity and recovery.

In order to determine structures of OsLu, the reaction mixtures resulting from hydrolysis using fungal and yeast β-galactosidases were purified using activated charcoal and analyzed by GC-MS as oxime TMS derivatives in a fused silica column coated with SPB-1 (cross linked methyl siloxane). Galactosyl-galactoses and galactosyl-fructoses as well as several trisaccharides were characterized in the mixtures [48].

GC-MS has also been used to quantify the *in vivo* ileal digestibility of synthesized oligosaccharides OsLu with DP≥2 and commercial GOS with DP≥3 [62]. For chromatographic analysis, carbohydrates were converted to their TMS oxime derivatives and analyzed by fused silica capillary column GC. The composition of a purified fraction of OsLu, obtained by hydrolysis of lactulose (Duphalac®) using β-galactosidase from *A. oryzae*, has also been determined by GC-FID of its TMS oxime derivatives. Before chromatographic analysis, oligosaccharide mixtures were treated with yeast (*Sacharomyces cerevisiae)* to eliminate monosaccharides. Carbohydrate analysis was performed in a ZB-5HT Inferno fused silica capillary column (5% phenyl and 95% dimethyl polysiloxane) [63]. OsLu consisted of a mixture of carbohydrates, which contained 28% monosaccharides; 12% lactulose; and 36% of prebiotic carbohydrates (17% disaccharides, 13% trisaccharides 5% tetrasaccharides and 1% pentasaccharides).

GC-FID has also been used to monitor isomerization of lactose to lactulose, from cheese whey permeate, using egg shell as catalysis. Analysis of de TMS

291 derivatives was performed using a commercial fused silica capillary column SPB-17
292 bonded cross linked phase [22]. Also, Ruiz-Matute et al. [41] quantified by GC-FID the
293 content of mono- (glucose, galactose, fructose and tagatose) and disaccharides
294 (allolactose, lactose, β(1→6)-galactobiose and sucrose) in commercial lactose-free UHT
295 milks and dairy products as their TMS oxime derivatives (**Figure 2**).
296
297 **4 Structural characterization**
298
299 Given that the structural features of carbohydrates, in terms of type of glycosidic
300 linkage, monosaccharide composition and molecular weight, determine their
301 functionality, the comprehensive structural elucidation of carbohydrates is required to
302 gain insight into the structure/function relationship. Among the different techniques
303 available for the structural characterization of carbohydrates, which include X-ray
304 crystallography, infrared and Raman spectroscopy, immunochemical labeling or
305 electron microscopy, MS and Nuclear Magnetic Resonance (NMR) spectroscopy are the
306 two most prevalent ones [64].
307 MS-based methods have a higher sensitivity than those based on NMR
308 spectroscopy and are favored when only a limited amount of material is available.
309 However, NMR spectroscopy is better suited for determining novel structures of
310 unknown carbohydrates and for the study of dynamical processes, since the structure at
311 atomic resolution can be elucidated [65]. Both techniques can also be coupled to
312 different high resolution separation techniques as HPLC, GC or CE, being these
313 hyphenated methods powerful tools for the structural elucidation of carbohydrates [66,
314 67].
315

316 *4.1 MS analysis*

317 The wide accessibility of hyphenated instrumental devices based on MS (HPLC-MS,
318 GC-MS, CE-MS), and the current availability of different ionization modes and mass
319 analyzers has greatly increased the potential of MS to characterize carbohydrates in the
320 last decades [68]. Electron impact (EI), electrospray ionization (ESI) and matrix-
321 assisted laser desorption/ionization (MALDI) are the most frequently ionization sources
322 used for the MS analysis of carbohydrates [69, 70].

323

324 *4.1.1 EI (GC-MS)*

325 EI is coupled on-line to GC for carbohydrate analysis and, thus, a combination of GC
326 retention data (linear retention indices, I^T) and MS data (relative abundances for
327 selected fragments) can provide useful information about ring size, the glycosidic
328 linkage of reducing end and other oligosaccharide structural features [71]. Thus, Sanz et
329 al. [72] used a multivariate statistical analysis to correlate the mass spectral data of the
330 TMS oximes of standard disaccharides with their structures, and this was successfully
331 applied to determine the composition of the disaccharide fraction obtained from the
332 hydrolysis and subsequent transgalactosylation of lactose with β-galactosidase from *K.*
333 *fragilis* by means of a quadrupole as mass analyzer working in EI mode at 70 eV**.**
334 Although mass fragments are common for most of the glycosidic linkages, these authors
335 were able to identify several galactobioses and galactosyl-glucoses having different
336 glycosidic linkages (from 1→2 to 1→6) from the relative intensity of a wide range of
337 characteristic fragment ions (**Figure 3**). Likewise, the presence of an α or a β glycosidic
338 bond was correctly predicted in 94% of cases according to the intensity of ion fragments
339 at *m/z* 243 or 204, respectively. Later on, a similar procedure was used for the structural
340 determination of the di- and trisaccharide fractions of GOS [57] or OsLu [48] using β-

galactosidases of different origin (*K. lactis, A. aculeatus* and *A. oryzae*). In the case of OsLu, the presence of a reducing fructose unit substituted in C1 or C3 gave rise to a characteristic fragment ion at *m/z* 307 [48]. Within the trisaccharide fraction, GC-MS data of 6'-galactosyl-galactobiose, 4'-galactosyl-lactose, 6'-galactosyl-lactose and 6'-galactosyl-lactulose, as well as of the galactosyl- and digalactosyl-glycerols produced during the transgalactosylation reaction due to the presence of glycerol as enzyme stabilizer in some commercial preparations, were also reported [48].

Methylation followed by GC-MS analysis has also been applied for the tentative determination of GOS as partially methylated alditol acetates by comparison with the profile of standard oligosaccharides [59]. These authors determined that the main structural elements in Vivinal® GOS were Gal-1 and 4-Gal-1, whereas for the reducing ends 4-Glc, 3-Glc, 6-Glc and 2-Glc were present in equivalent abundances.

4.1.2 ESI (HPLC-MS^n)

ESI has extensively been used to analyze oligosaccharides and can be employed without any previous separation technique to analyze simple oligosaccharide mixtures. However, for the characterization of complex oligosaccharides mixtures, as the case of GOS, ESI is commonly coupled to LC. In oligosaccharide characterization studies, the most commonly used mass analyzers are quadrupole (Q), time-of flight (TOF), quadrupole ion trap (QIT), linear ion trap (LIT/LTQ) and Orbitrap. Additionally, tandem systems composed of two or more coupled analyzers of the same or different types, such as triple quadrupole (QqQ) or the quadrupole coupled to time-of-flight (Q-TOF), are employed with the aim of gaining more information on the oligosaccharides structural features [68].

Q and microTOF provided with an ESI source operated under positive polarity coupled to HPLC on graphitized carbon and hydrophilic interaction columns, respectively, were useful to determine the DP (from di- to hexasaccharides) of OsLu [48] and GOS (from tri- to heptasaccharides) [73]. An unusual coupling technique such as HPAEC-MS using an LTQ with an ESI source operated in both positive and negative mode was used for the determination of the DP (from di- to tetrasaccharides) and quantification of GOS [59]. In this case, removal of salts present in the mobile phase carried out by a suppressor that exchanges Na^+ with H^+ ions is essential prior to MS detection. However, in this work the authors did not make use of the capacity of ion traps to perform multi-stage fragmentation and gain insight on oligosaccharide structures, and the characterization was accomplished by NMR as it will be explained below. GOS synthesized from lactose and previously purified by LC were analyzed by Neri et al. [74] using ESI(+) and MS^n (n = 2,3) on a LTQ mass spectrometer. The initial ESI-MS analysis determined the presence of di-, tri- and tetrasaccharides; whilst the ESI-MS/MS experiments allowed the detection of major fragment ions at *m/z* 305, 275 and 245 in the disaccharide fraction which were attributed to the cross ring fragmentation of two glucose units linked by a α(1→6) type linkage [69]. Other major fragment ions at *m/z* 467 and 407 in the trisaccharide fraction were detected; these were formed by the loss of 60 Da ($-C_2H_4O_2$) and 120 Da ($-C_4H_8O_4$) fractions, suggesting the presence of a α(1→4) linkage at the reducing end. MS^3 analyses also served to determine the presence of an α(1→6) linkage in the trisaccharide fraction**,** the α(1→4) linkage at the reducing end and two other α(1→6) linkages in the tetrasaccharide fraction. This knowledge was expanded by Hernández-Hernández et al. [47], who carried out a comprehensive elucidation of the structure of di- and trisaccharides of three different commercial GOS by HILIC-ESI(+)-MS^n using a linear ion trap as mass

analyzer. These authors described characteristic fragment ions for (1→3) (i.e., at *m/z* 347, 275, 203), (1→4) (at *m/z* 305, 203 and 347) and (1→6) type linkages (at *m/z* 305, 275 and 245) in the disaccharide and trisaccharide fractions. In the case of tetrasaccharides only some glycosidic linkages were tentatively assigned and the monomer composition could not be determined. This was attributed to the decrease of the abundance of fragment ions derived from cross-ring fragmentation with increasing number of MS cycles, as well as to the existence of multiple coelutions [47].

4.1.3 MALDI

Unlike EI and ESI, MALDI is not coupled directly to a GC or HPLC system. In MALDI the analyte is embedded in an excess of an appropriate low molecular weight matrix molecule and then desorbed and ionized by a short laser pulse. Depending on the oligosaccharide structure and the molecular weight, different matrices have been used, with 2,5-dihydroxybenzoic acid and 2,4,6-trihydroxyacetophenone being the most commonly used [68]. Indeed, the former has been successfully used for the analysis of GOS synthesized using active β-galactosidase inclusion bodies-containing *Escherichia coli* cells [75]. MALDI-TOF data revealed that the GOS were mainly composed by the trisaccharide fraction, although *m/z* values indicative of the presence of tetra- and disaccharides were also detected. Rodriguez-Colinas et al. [37, 39] and Urrutia et al. [76] used the same matrix (i.e., 2,5-dihydroxybenzoic acid) to determine by MALDI-TOF the presence of several GOS (di-, tri- and tetrasaccharides) synthesized either by ethanol-permeabilized *K. lactis* cells or by β-galactosidases from *B. circulans* and *A. oryzae.*

In an elegant study, Barboza et al. [77] demonstrated the utility of MALDI with Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) to

415 determine the oligosaccharide composition of GOS syrup preparations that contained
416 oligosaccharides with DP ranging from 3 to 11. Indeed, the oligosaccharides with the
417 highest DP, i.e. 10 and 11, could be only detected after adjusting the voltage of the
418 MALDI-FT-ICR quadruple ion guide. Furthermore, following a previous fractionation
419 of the GOS syrup accomplished by SEC, oligosaccharides with DP up to 15 were also
420 detected in the Bio-Gel P-2 excluded fraction. In addition, these authors developed a
421 microplate protocol for growing four different bifidobacterial strains on purified GOS,
422 followed by MALDI-FT-ICR analysis to profile the specific oligosaccharide species
423 consumed. Results indicated that GOS with DP ranging from 3 to 8 were preferentially
424 consumed by the infant-borne isolates, i.e. *B. longum* subsp. *infantis* and *B. breve*,
425 whilst the other two tested strains (*B. adolescentis* and *B. longum* subsp. *longum*)
426 exhibited more differential consumption of select DPs. Overall, these findings
427 demonstrated that MALDI-FT-ICR is a rapid-through-put tool for the comprehensive
428 profiling of oligosaccharides in complex GOS mixtures.
429
430 *4.2 NMR spectroscopic analysis*
431 Currently, *de novo* structural elucidation of unknown compounds in solution can be
432 only achieved by the exhaustive use of 1D and 2D homo- and heteronuclear NMR
433 spectroscopy assisted by other spectroscopic methods [67]. This is particularly
434 important in the case of oligosaccharides by bearing in mind the limited number of
435 commercial standards. Likewise, in conjunction with molecular modeling and molecular
436 dynamics simulations, NMR spectroscopy has the capacity to determine the 3D
437 structures of oligosaccharides [65].
438 Concretely, NMR spectroscopy has been used in the characterization of several
439 oligosaccharides derived from lactose and/or lactulose. The analyzed oligosaccharide

should be present in high purity because of the signal richness of NMR spectra, indicating that a purification step prior to NMR based structure analysis should be accomplished. This fact can be an important drawback in the case of GOS mixtures since its enzymatic synthesis normally leads to complex mixtures of structurally similar carbohydrates as it was indicated in the section 2. Consequently, the uses of advanced analytical techniques, which can provide an adequate chromatographic resolution, are very often required. This was the case for the characterization of several GOS di- and trisaccharides which were previously isolated by preparative HILIC and then identified on the basis of their methylation analysis and ^{1}H NMR and/or ^{13}C NMR data [59]. Rodriguez Colinas et al. [37, 39] also purified GOS by semipreparative HILIC to further successfully elucidate the structure of the major synthesized GOS products, i.e. 6-galactobiose, allolactose, 3-galactosyl-glucose, 6'-galactosyl-lactose, 4'-galactosyl-lactose and the tetrasaccharide β-D-Gal-(1→4)-β-D-Gal-(1→4)-β-D-Gal-(1→4)-Glc, using a combination of 1D (^{1}H, ^{13}C) and 2D (COSY, TOCSY, NOESY, HSQC) NMR techniques. Neri et al. [74] characterized similar trisaccharide structures, i.e. 6-galactosyl-lactose and 6'-galactosyl-lactose, and the tetrasaccharide β-D-Gal-(1→6)-β-D-Gal-(1→6)-β-D-Gal-(1→4)-Glc according to 1D (^{1}H, ^{13}C) and 2D (COSY, HSQC and HMBC) NMR experiments.

Two GOS trisaccharide isomers, 6'-galactosyl-lactose and 3'-galactosyl-lactose, enzymatically synthetized and, subsequently, acetylated and purified by silica gel column chromatography were structurally characterized by 1D (^{1}H, ^{13}C) and 2D (COSY, HSQC and HMBC) NMR analysis [78]. These authors indicated that acetylation simplified the analysis of NMR spectra as it blocked the free hydroxyl groups of sugar ring.

Martinez-Villaluenga et al. [9] also performed a comprehensive characterization of two novel trisaccharides, 6′ galactosyl-lactulose and 1-galactosyl-lactulose, obtained by transglycosylation of lactulose with the β-galactosidase from *K. lactis*, and which were chromatographically purified by HPLC and then fully characterized by 1D (^{1}H, ^{13}C, and 1D TOCSY) and 2D (gCOSY, TOCSY, ROESY, gHSQC, and gHMBC) NMR studies. Similar chromatography and NMR approaches for the structural elucidation of two potential bioactive oligosaccharides, 2-α-D-glucopyranosyl-lactose and lactulosucrose obtained from the *L. mesenteroides* B-512F dextransucrase-catalyzed reactions using lactose [53] or lactulose [23] as acceptors, have also recently been carried out.

5 Bioactivity

Although prebiotics present in foods have been consumed since prehistoric times, during the last decades, special attention has been paid on these functional ingredients due to their bioactive effect on intestinal microbiota which, as it is known, is narrowly related to the health of the host. Among the recognized prebiotics, GOS has emerged as a practical and efficient choice to be incorporated in several foodstuffs to positively modify the gut microbiota due to the wide number of studies related to their benefits on human health [79]. GOS benefits the health of host by two principal mechanisms; one is related to the stimulation of the growth of beneficial bacteria, lactobacilli and, mainly, bifidobacteria in the gut [80, 81], and the other is related to the production of Short Chain Fatty Acids (SCFA) (acetate, propionate, butyrate) during fermentation of carbohydrates. These metabolism products of bacteria play an important role in

488 biochemical and physiological processes not only in the large gut but also in remote
489 body places [82].

490 The positive effects of GOS on human health have been largely reviewed in the
491 literature, however, much less is known on the benefits of the recently described OsLu.
492 Given their structural similarity, it is presumable that both types of compounds also
493 share some bioactive properties. This review includes the main effects of GOS and
494 OsLu to date described.

495

496 *5.1 Digestibility*

497 As it is known, one of the conditions for oligosaccharides to be considered as prebiotics
498 is the fact that they must resist the small intestinal digestion to exert the positive effect
499 on the large intestine. Ohtsuka et al. [83] observed that only a small proportion of the
500 4'-galactosyl-lactose was *in vitro* digested by a homogenate of intestinal mucosa of rats.
501 Torres et al. [84] revised, among other properties, the digestibility of GOS. Thus, *in*
502 *vitro* and *in vivo* assays pointed out that more than 90% of GOS are stable to digestive
503 enzymes and achieve the colon to exert their effect. *In vitro* trials showed that GOS with
504 DP≥3 are resistant to salivary enzymes. *In vivo* human studies have been focused on the
505 hydrogen breath test, which is dose-dependent; thus, 15-35 g/d unequivocally
506 demonstrated that GOS were fermented but not digested, however, with 10 g/d odd
507 results were obtained. In rats, Hernández-Hernández et al. [85] pointed out a higher
508 resistance of OsLu as compared to GOS to gastrointestinal digestion and absorption in
509 the small intestine, probably due to the β(1→4) linkage between galactose and fructose
510 at the reducing end of the OsLu molecules.

511

512 *5.2 Prebiotic effect*

513 Among the different biological activities of GOS, the principal is the prebiotic effect.
514 According to Gibson et al. [86], prebiotic is an ingredient that when is selectively
515 fermented gives rise to specific changes in the composition and in the activity of
516 intestinal microbiota which benefit the health and well-being of the host [79].

517 A great number of studies recognize GOS as prebiotic [79, 86-89]. The prebiotic
518 effect of GOS can be tested *in vitro* using a simple fermentation batch system with
519 human fecal culture without pH control [90], a three-stage continuous model of the
520 human gut [91-93] and *in vivo* with randomized, double-blind, crossover, placebo-
521 controlled intervention study in humans [79]. **Table 1** summarizes some studies carried
522 out in the last 5 years on the prebiotic effect of GOS in humans.

523 With respect to individual OsLu, recent *in vitro* studies showed that they can be
524 fermented by different strain of *Bifidobacterium*, *Lactobacillus* and *Streptococcus*
525 [103]. OsLu with DP≥3 presented bifidogenic activity in human fecal slurries [104].
526 Lately, Cardelle-Cobas *et al.* [105] assessed their prebiotic properties considering the
527 growth of *Bifidobacterium, Lactobacillus, Enterococcus, Eubacterium, Bacteroides,*
528 *Clostridium* and *Atopobium* and the production of SCFA in mixed fecal microbiota. *In*
529 *vivo* assays showed that, in rats feed with 1% (w/w) GOS or OsLu after 14 days, the
530 balance between beneficial bacteria (bifidobacteria, lactobacilli and *Eubacterium*
531 *rectale*) and "less desirable" bacteria (bacteroides and clostridia) was better for OsLu
532 than for GOS [85]. In addition, a selective increase of *Bifidobacterium animalis* counts
533 in the cecum and colon of these rats was observed [106]. All these findings highlight

that the new oligosaccharides OsLu may constitute an alternative as prebiotics to the original disaccharide lactulose and also to GOS. Further *in vivo* studies carried out with humans are currently underway in order to definitively establish their prebiotic properties.

5.3 Modulation of immune system and effect on allergy

Intestinal microorganisms and SCFA production during saccharolytic metabolism can positively affect the immune responses and protect against the development of inflammatory diseases [82]. Particularly, during fermentation of GOS, the produced butyrate and propionate have been demonstrated to participate in several mechanisms related to immune modulation such as stimulation of apoptosis and suppression of cytokines, among others [80]. This effect could be age-dependent; thus, in artificially reared newborn rats with GOS and fructooligosaccharides (FOS) supplementation, Barrat et al. [107] found that the SCFA production during fermentation could impair the intestinal barrier function.

The intestinal mucosa has large amounts of secretory immunoglobulin A (s-IgA), which has a protective role against the adherence and invasion by harmful bacteria and viruses [88]. It has been reported the positive effect of enteral administration of GOS in the maintenance of intestinal barrier function in rats with severe acute pancreatitis, partially attributed, among other factors, to stimulation of mucous s-IgA that increased from 49 to 66.7 μg/g as compared to rats with non-supplemented feeding [108]. In overweight adults the intake of Bimuno® GOS, a commercial mixture of GOS,

556 increases faecal s-IgA and decreases faecal calprotectin and plasma C-reactive protein
557 [95].

558 Vulevic et al. [109] carried out a trial with healthy elderly volunteers and
559 evidenced a decreased secretion of proinflammatory cytokines (IL-6, IL-1β and TNF-α)
560 and an improvement in NK cell activity and increased secretion of the anti-
561 inflammatory cytokines, IL-10.

562 Inadequate immune response to the normal microbiota gives rise to
563 inflammatory processes such as Crohn's disease and ulcerative colitis. Although some
564 results are contradictory, the intake of prebiotics can positively affect these diseases.
565 Holma et al. [110] did not find reduction in the inflammatory process in colitis induced
566 rats which had intake 4 g of GOS/kg of body mass per day and they attributed these
567 results to the animal model. In mice treated with the pathogen *Helicobacter hepaticus,*
568 Gopalakrishnan et al. [111] suggested that GOS could be a novel approach for
569 inflammatory bowel disease since these oligosaccharides significantly reduced the
570 severity of colitis (**Figure 4**) together with an increase in the percentage of NK cells.

571 Bimuno® GOS has shown potential for ameliorating the symptoms of irritable
572 bowel syndrome in human (**Table 1**). Thus, in addition to the selective increase of
573 bifidobacteria, side effects such as flatulence, bloating, stool consistency, anxiety and a
574 subjective global assessment of severity were improved in subjects who intake GOS.
575 Although the mechanisms of the effect are unknown the authors draw parallels with a
576 study on the effect of bifidobacteria that favored the normalization of the aberrant IL-
577 10/IL-12 ratio in peripheral blood of inflammatory bowel syndrome patients [96].

578 An interesting although preliminary study in nonsymptomatic highly active
579 antiretroviral therapy-naive HIV-1-infected adults (**Table 1**), suggests that, a mixture of

580 GOS/lcFOS/pAOS (long-chain FOS/pectin-derived acidic oligosaccharides) (9/1/10) at
581 a dose of 15 or 30 g/d, improves NK cell cytolytic activity, as well as reduces of HIV-1-
582 induced immune activation [100].

583 With respect to OsLu, only one *in vitro* study using intestinal epithelial cells
584 (Caco-2 and HT29-MTX) has investigated the modulation of immune system and an
585 increase of anti-inflammatory cytokines (IL-6, IL-10) and reduction of pro-
586 inflammatory factors (TNF-a, IL-1β) was observed [112].

587 Several studies have indicated that could have evidence for a link between the
588 colonic microbiota and allergy. In developed countries an increase in allergic diseases
589 has been observed and it is attributed to a less microbial exposure in early childhood,
590 among other hypothesis. Allergic children have less lactobacillus and bifidobacteria and
591 prebiotics might favor the development of beneficial microbiota. The effects of
592 prebiotics on allergy have been investigated in a preventive setting in animal and human
593 assays [113, 114]. In infant formula, a mixture of GOS/lcFOS has been studied due to
594 prebiotic effect similar to human milk [115, 116]. This prebiotic infant formula
595 consumed during the first 6 months of life (8 g/L) has also shown a reduction in the
596 incidence of atopic dermatitis and infectious episodes, not only in this period [117], but
597 also during the first 2 years of life [118]. In addition, during the 6-month of treatment,
598 an increase in bifidobacteria levels in the feces and a fall in the levels of total IgG1,
599 IgG2, IgG3, and IgE was observed [117].

600 Other study, with 414 infants fed with a formula with prebiotic mixture
601 supplementation GOS/lcFOS/pAOS (9/1/2, 8 g/L) showed the effectiveness as primary
602 prevention of atopic dermatitis. Furthermore, the prebiotic intervention group had a

603 smaller number of upper respiratory tract infections, fever episodes and courses of
604 antibiotic therapy [119].

605 The role of symbiotics in allergy prevention has been recently reviewed [114]. In
606 a clinical human study, near of 1,000 mothers with infants at high risk for allergy were
607 randomized to receive a capsule containing freeze-dried probiotic mixture surplus 0.8 g
608 of GOS during the last month of pregnancy and their infants to receive it from birth
609 until age 6 months. After 2 years, the treatment seemed to increase resistance to
610 respiratory infections and tended to reduce IgE-associated to eczema, but showed no
611 effect on food allergy, asthma and rhinitis [120]. However, the anti-allergic effect was
612 not sustained over 5 years [121]. In other study, a symbiotic mixture of bifidobacteria
613 plus GOS/lcFOS (8 g/L) showed no detectable effect on plasma levels of the analyzed
614 atopic disease markers (of IL-5, IgG1, IgG4, and others) [122].

615

616 *5.4 Antipathogenic effect*

617 GOS and other prebiotics may directly inhibit intestinal infections by enteric pathogens
618 due to their capacity to mimic the places of binding in the surface of gastrointestinal
619 epithelial cells. Tzortzis et al. [92], in an *in vitro* assay with Bimuno® GOS, showed a
620 strong inhibition in attachment of enterohepatic *E. coli* and *Salmonella enterica*
621 serotype Typhimurium to HT29 cells. Shoaf et al. [123] found the highest adherence
622 inhibition of *E. coli* E2348/69 on Hep-2 and Caco-2 cells with GOS as compared to
623 FOS, inulin, lactulose and raffinose. The anti-adhesive ability of GOS on *Cronobacter*
624 *sakazakii*, an opportunistic pathogen implicated in serious neonates infections, has been
625 also demonstrated in Hep-2 human cell lines [124].

In vivo assays carried out with BalbC mice fed with Bimuno® GOS before induction of salmonellosis demonstrated that although the pathogen was recovered in the feces, no disease symptomatology was observed [125], the protective effect being attributed to GOS [126]. Lately, in murine studies, Searle et al. [127] indicated that the low molecular weight fractions of Bimuno® GOS may be the primary stimulant of both pro- and anti-inflammatory cytokines by macrophages that can promote antigen presenting cell recruitment enhancing pathogen phagocytosis. Drakoularakou et al. [128] in a study in 159 healthy volunteers assessed the capacity of Bimuno® GOS (5.5 g/d) in reducing travelers' diarrhea, probably ascribed to the inhibition of pathogen adhesion.

Infant formula supplemented with prebiotic (GOS/lcFOS, 9/1), have shown to exert a protective effect against respiratory infections during the first year of age in children [118, 129]. A similar effect was observed with consumption of infant formula with symbiotics (4 probiotic species with 0.8 g/day of GOS), that reduced frequency of respiratory infections during the first 2 years of life [120].

Gastrointestinal dysfunction and cold/flu symptoms due to acute psychological stress related to academic exams were reduced by means of commercial GOS powder (Purimune®) supplementation in healthy university students [130].

5.5 Mineral absorption

Several investigations in rats and in humans have reported that GOS fermentation in the gut improves the absorption of minerals, mainly Ca [131-135]. One of the possible involved mechanisms is based on the action that, during fermentation of GOS, the

649 production of SCFA in the large intestine results in a lowering of pH. Thus, the mineral
650 solubility and their absorption across the epithelial cells of colon and cecum can be
651 increased [136]. Other mechanism is related to the increase in the available surface for
652 absorption due to the proliferation of epithelial cells, as studied in animal and human
653 assays [137, 138].

654 Recently, in a study carried out in rats with hypochlorhydria, Takasugi et al.
655 [139] have shown that the combination of fermented milk with GOS improves the
656 retention of Ca, Fe and Zn and decreases the urinary excretion of P, and, moreover, this
657 combination also increased bone strength. An increase in the absorption of Ca was also
658 observed in a study with thirty-one healthy adolescent girls who daily intake 5 g of GOS
659 in smoothed drinks during three weeks [94].

660

661 *5.6 Effect on lipid metabolism and related metabolic disorders*

662 The effect of GOS on the lipid metabolism is still unclear and it has been scarcely
663 studied. Dokkum et al. [140] administrated to healthy humans 15 g/d of Vivinal® GOS,
664 FOS and inulin and no effects on glucose absorption and serum lipids were detected.
665 Total cholesterol (TC) and LDL cholesterol levels did not change in infants feed with
666 GOS and FOS in the infant formula [141]. TC and HDL cholesterol were not affected in
667 healthy elderly people after the intake of 5.5 g of Bimuno® GOS [108]. However, very
668 recently, Vulevic et al. [95] have demonstrated that the same GOS mixture significantly
669 reduces TC and triglycerides, while having no effect on the levels of LDL and HDL
670 cholesterol in overweight subjects with metabolic syndrome. In this sense, as
671 oligosaccharides are not viscous fibers, they unlikely decrease the absorption of

672 cholesterol and other mechanisms related to the intestinal microbiota can be involved.
673 Among them, enzymatic deconjugation of bile salts by bacteria, incorporation of lipids
674 into bacteria cellular membranes during growth, conversion into coprostanol and fecal
675 excretion and inhibition of cholesterol synthesis in the liver through the production of
676 SCFAs have been proposed as responsible for the observed beneficial effects of GOS
677 [142].

678 On the other hand, the occurrence of overweight and its negative effects on
679 human health worries to sanitary authorities and consumers. During the recent years,
680 obesity has been associated with changes in the microbiota, reduced bacterial diversity
681 and altered representation of genes and metabolic pathways. Overduin et al. [143] have
682 studied the effects of long-term (3-week) and acute (4 h) Vivinal® GOS consumption on
683 parameters of energy balance in young adult male rats. The GOS-fed rats showed
684 increased caecal and reduced fat-pad weight and increased gene expression of the
685 satiety-related peptides, PYY (1.7 fold) and proglucagon (3.5 fold).

686

687 *5.7 Other beneficial effects*

688 In addition to the above mentioned, GOS can present other positive effects that should
689 be taken into account. Prebiotic effects also modulate stooling pattern, pH (due to
690 increase of SCFA levels), consistency and frequency. In infant feed with formulae
691 supplemented with 2.4-4 g/L GOS the stools were similar to those of breast-fed infants
692 [144, 145]. Addition of polidextrose and GOS to a follow-on formula was well tolerated
693 and induced a pattern of more frequent and softer stools in toddlers [146]. Lamsal [147]
694 reviewed that in women who had a tendency to constipation a 5 g/d dose of GOS, for 1

695 week, improved defecation frequency, similarly in healthy volunteers consuming GOS-
696 supplemented yoghurt (9–15 g) daily for 2–3 weeks.

697 With respect to prevention of colon cancer, the increase in the levels of
698 bifidobacteria and lactobacillus can be also related to anti-mutagenic and anti-tumor
699 properties, increasing the protection against cancer. Moreover, the enhanced production
700 of SCFA after fermentation can directly or indirectly affect enterocyte proliferation,
701 carcinogenesis, enzyme activities and the production of nitrogenous metabolites, all
702 related with colon cancer [148]. In spite of this positive hypothesis no concluding
703 studies have been carried out. Macfarlane et al. [88] reviewed the effect of prebiotics in
704 cancer and they showed that GOS only decreased the incidence of aberrant crypt
705 multiplicity in rats. In humans, reduced activities of genotoxic enzymes (β-
706 glucuronidase) produced by colonic micro-organisms have been proved and this fact has
707 been linked to colon cancer.

708 Other benefit of GOS could be their potential positive effect on neurological
709 diseases. In $SOD1^{G93A}$ mouse model, Song et al. [149] have suggested that GOS (even
710 in a prebiotic yogurt) might have therapeutic potential for amyotrophic lateral sclerosis
711 since their administration significantly delayed the disease beginning and prolonged the
712 lifetime due to the increase of vitamin absorption and reduction of homocysteine,
713 among other mechanisms.

714

715 **6 Conclusions**

716

717 Although the synthesis of bioactive carbohydrates from lactose has been
718 extensively investigated, recent research has focused on developing new bioactive

carbohydrates that could result in commercial products. The growth of cheese production leads to a steady increase availability of lactose-rich permeates so that the development of new ways to use lactose is a constant challenge for researchers.

In recent years the synthesis, characterization and biological activities of new lactose derivatives have been studied with the aim of obtaining compounds with new or improved bioactivities. However, more research is needed to extend the knowledge of structural characterization, and bioactive properties, not only to understand the mechanisms of bioactivity in the characterized new compounds but also for the future design of more effective multifunctional lactose derived oligosaccharides. Among these studies, clinical assays are essential to verify its usefulness in humans.

Finally, the development of novel bioactive oligosaccharides will be of use only if they are formulated into foods and placed in the market. For achieving that purpose, the manufacture of this type of oligosaccharides on a commercial scale requires technically feasible and economically viable processing methods. Likewise, it is necessary to gain further knowledge on the physico-chemical and organoleptic properties of potential bioactive oligosaccharides in the context of real food products, as well as on their stability properties during food processing and storage.

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Figure legends

Figure 1. HPAEC-PAD carbohydrate profiles of oligosaccharides formed from transgalactosylation of lactulose using a β-galactosidase from *A. aculeatus* at pH 6.5, 60 °C, 650 g/L of lactulose, and 16 units/mL of enzyme after 7 h of reaction. Identified compounds: (1) galactose; (2) fructose; (3) β-D-Gal-(1→6)-Gal; (4) lactulose; (5) β-D-Gal-(1→6)-β-D-Gal-(1→4)-β-D-Fru (6′-galactosyl-lactulose); (6) β-D-Gal-(1→4)-β-D-Fru-(1→1)-β-D-Gal (1-galactosyl-lactulose); (7) oligosaccharides with DP ≥ 3. Reprinted with permission from Cardelle-Cobas et al. Copyright (2008) American Chemical Society.

Figure 2. GC-FID profile of TMS oxime derivatives of carbohydrates present in a commercial Lactose Free-UHT milk. (1a, 1b) Tagatose; (2a, 2b) fructose; (3) glucose; (4) galactose; (5a, 5b) lactose; (6a, 6b) allolactose; (7a, 7b), 6-galactobiose; (I.S.) internal standard.

Figure 3. Mass spectra of disaccharide TMS oximes of kojibiose (A), turanose (B), maltose (C), leucrose (D), 6-galactobiose (E) obtained by EI mode at 70 eV. Reprinted with permission from Sanz et al. Copyright (2002) Springer.

Figure 4. Representative images (200 × magnification) corresponding to colonic and cecal crypts before and after infection by *H. hepaticus* in mice feed with/without GOS (arrows denote inflammatory infiltrate). Reprinted with permission from Gopalakrishnan et al. Copyright (2012) American Society for Nutrition.

1056 **Table 1.** Recent human studies designed to determine the prebiotic effect of GOS.

Prebiotic	Subject (n)	Dose	Duration	Effect	References
Vivinal® GOS	Healthy adolescent girls (31)	5 or 10 g/d	9 weeks	Increase of: fecal bifidobacteria counts with 5 g/d GOS dose	Whisner et al. 2013 [94]
Vivinal® GOS	Volunteers aged 50–81 years (39)	8 g/d	6 weeks	*In vivo:* a significant bifidogenic effect *In vitro:* a saccharolytic effects with increase of bifidobacteria and lactobacilli counts and butyrate level	Walton et al. 2012 [93]
Bimuno® GOS	Overweight adults (45)	5.5 g/d	24 weeks	Increase of: bifidobacteria counts and s-IgA level Decrease of: fecal calprotectin, plasma C-reactive protein, insulin, total cholesterol and triglycerides level	Vulevic et al. 2013 [95]
Bimuno® GOS	Irritable bowel syndrome patients (44)	3.5 or 7 g/d	12 weeks	Increase of: fecal bifidobacteria counts and amelioration of symptoms of irritable bowel syndrome	Silk et al. 2009 [96]
Purimune® GOS	Healthy human volunteer aged 19-50 years (18)	2.5, 5 and 10 g/d	3 weeks per dose	Increase of: Actinobacteria, mainly *Bifidobacterium;* Firmicutes in few individuals. Response varied between individuals, reversible, and in accordance with dose	Davis et al. 2011 [97]
GOS/FOS (9/1)	Infants born from hepatitis C virus-infected mothers (20)	8 g/L	6 months	Increase of: fecal bifidobacteria and lactobacilli counts during the first year	Salvini et al. 2011 [98]
GOS/FOS (9/1)	Healthy neonates (110)	8 g/L	28 days	Fecal bifidobacteria levels and soft stools, comparable to those found in breast-fed infants	Veereman-Wauters et al. 2011 [99]
GOS/FOS/pectin hydrolyzate-derived acidic oligosaccharides (9/1/10)	Highly active antiretroviral therapy-naive HIV-1-infected adults (57)	15 or 30 g/d	12 weeks	Increase of: bifidobacteria counts. Decrease of: *Clostridium coccoides/Eubacterium rectale* and pathogenic *C. lituseburense/C. histolyticum* cluster counts	Gori et al. 2011 [100]
Polydextrose + GOS (ratio 1/1)	Term infants (230)	4 g/L	60 days	Bifidogenic effect and soft stools similar to breast milk	Scalabrin et al. 2012 [101]
Probiotics and GOS	Healthy men (18)	3.8 g/d	2 weeks	Increase of: bifidobacteria counts Decrease of: β-glucosidase activity	Kekkonen et al. 2011 [102]

Figure 1

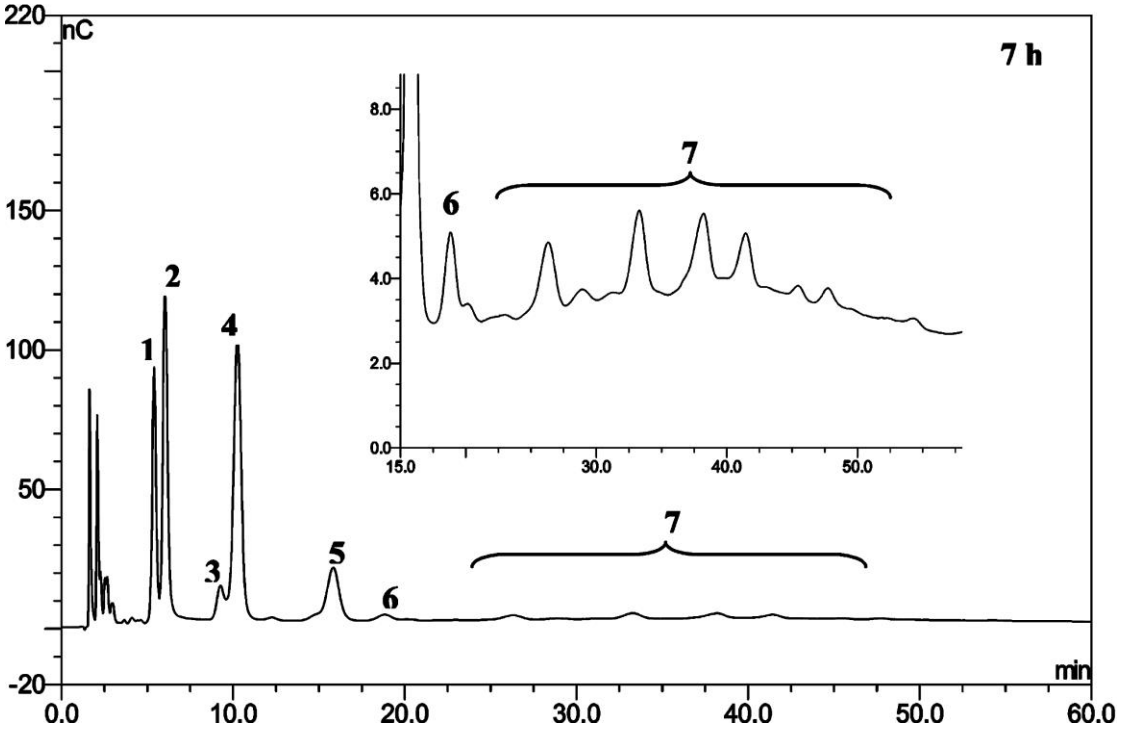
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Figure 2

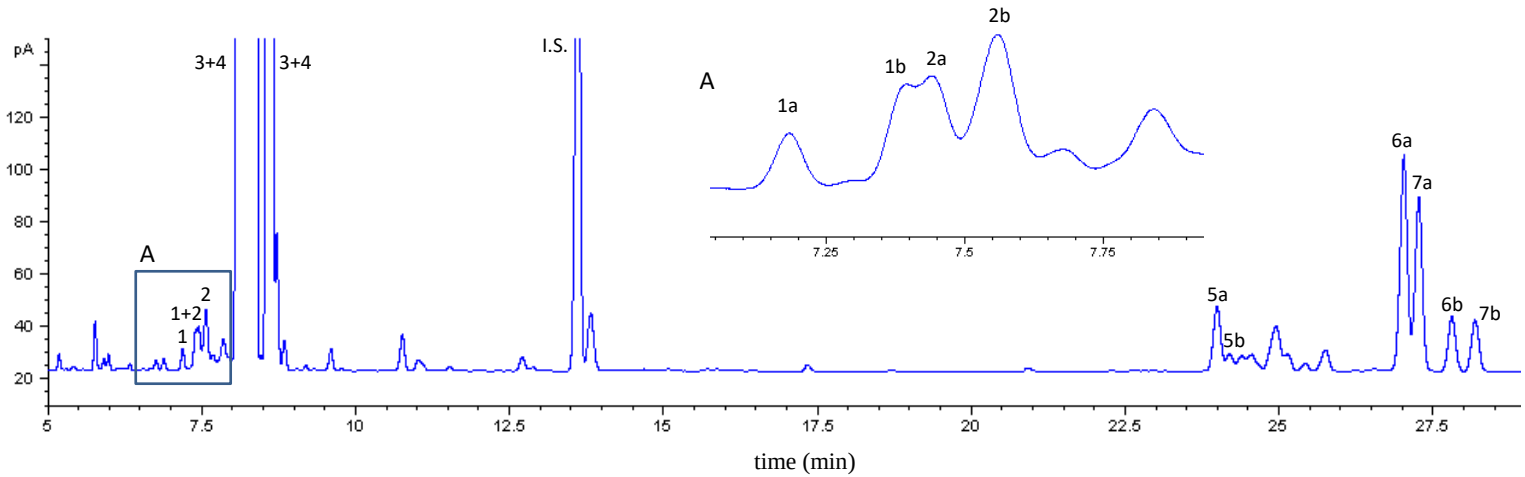
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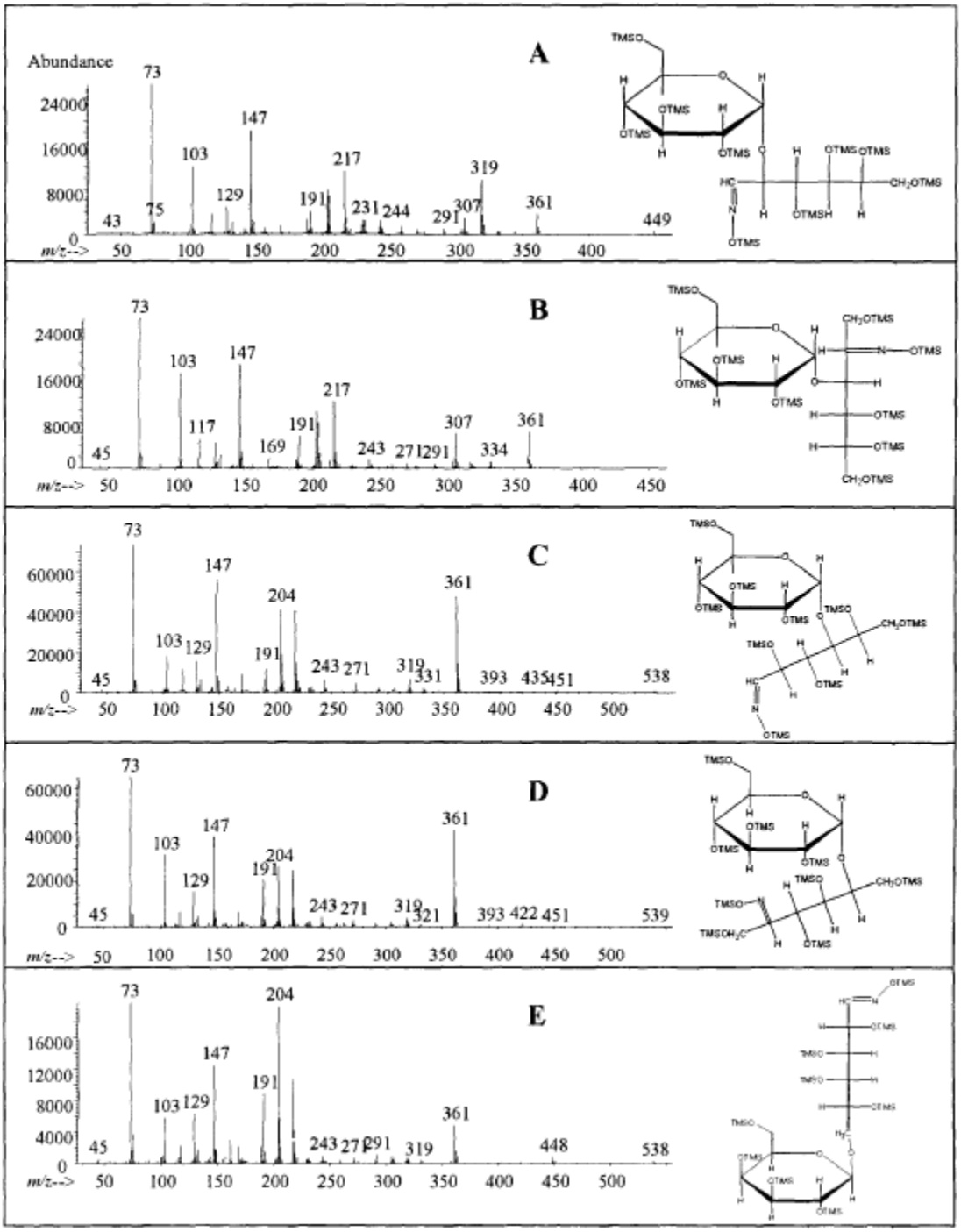
Figure 3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291
307
361
43
75
449
m/z-->
B
73
147
103
217
117
191
307
361
169
243
271
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334
45
C
73
147
204
361
103
129
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331
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435
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D
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448
538
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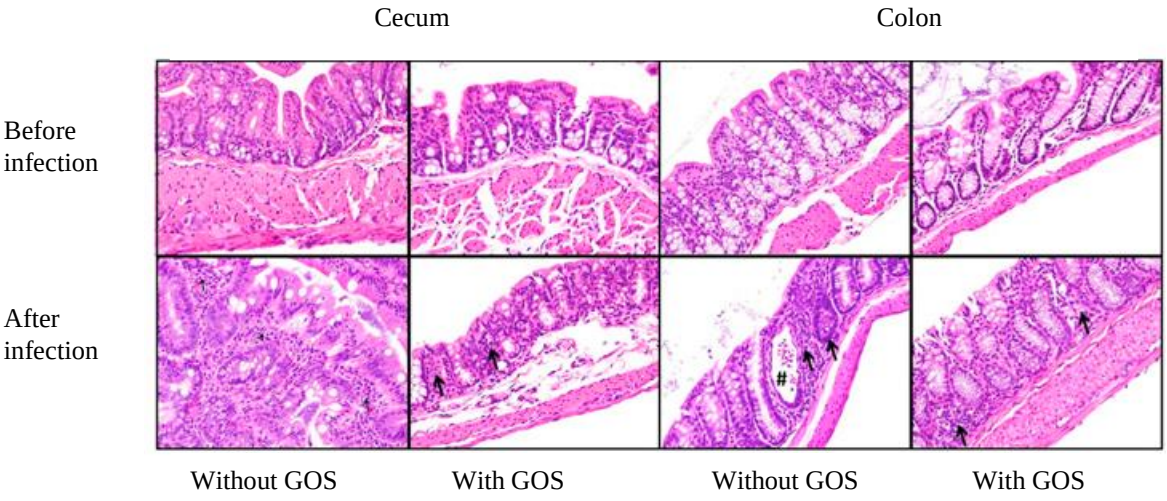
Figure 4


Cecum
Colon
Before infection
After infection
Without GOS
With GOS
Without GOS
With GOS