STABILITY OF OLIGOSACCHARIDES DERIVED FROM LACTULOSE DURING THE PROCESSING OF MILK AND APPLE JUICE

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Abstract

The scientific evidence on the bioactivity of oligosaccharides from lactulose (OsLu) has encouraged us to study their physicochemical modifications during the processing of real matrixes such as milk and apple juice. Carbohydrate fraction with degree of polymerization ≥ 3 was stable in milk heated at temperatures up to 100°C for 30 min and in apple juice heated up to 90°C for 15 min. An assessment of the initial steps of Maillard reaction in heated milk pointed out a higher formation of furosine in milk with OsLu as compared to its counterpart without OsLu, due to a higher presence of galactose. The organoleptic properties of juice with OsLu were acceptable and similar to those of apple juice with commercial galactooligosaccharides. The results here presented demonstrated that OsLu can be used as prebiotic ingredient in a wide range of functional food, including those destined for diabetic and intolerant to lactose.

Keywords: oligosaccharides derived from lactulose, prebiotic, milk, apple juice, processing.

1. Introduction

Nowadays, the consumers demand foods, with high quality at a reasonable price, that possess essential nutrients, adequate organoleptic properties and positive effects on certain physiological functions of the body, contributing, in this way, to improve their health and well-being. In this context, there is a growing interest towards functional ingredients, those related to gastrointestinal function, namely prebiotics, being one of the most important, because of their effect in the gut, where many serious diseases (diarrhea, inflammation, cancer, etc.) can take place. Moreover, there is a growing recognition that events taking place in the intestine influenced by the microbiota have major consequences for human health (Lamsal, 2012).

A range of prebiotics with various origin and chemical properties are currently used; among them, inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS) and lactulose are recognized as established prebiotics with several applications in the food industry (Patel, & Goyal, 2012). Lactulose (4-O-β-D-galactopyranosyl-D-fructose) is a lactose derived carbohydrate and it is resistant to hydrolysis by enzymes of small intestine and can reach the proximal colon where it is selectively fermented by bifidobacteria and lactobacilli producing carbon dioxide, hydrogen and short-chain fatty acids (Olano, & Corzo, 2009). On the basis of the fact that lactulose does not achieved the distal region of the colon, our research group obtained and exhaustively characterized oligosaccharides derived from lactulose (OsLu) with degree of polymerization ≥ 3 (Cardelle-Cobas, Corzo, Villamiel, & Olano, 2008; Cardelle-Cobas, Martinez-Villaluenga, Villamiel, Olano, & Corzo, 2008; Martinez-Villaluenga, Cardelle-Cobas, Olano, Corzo, Villamiel, & Jimeno, 2008; Cardelle-Cobas, Corzo, Martinez-Villaluenga, Olano, & Villamiel, 2011; Hernandez-Hernandez, Montanes, Clemente, Moreno and Sanz, 2011). These compounds, due to their larger size, might

be fermented in the distal portions of the gut and, thus, exert its beneficial effects there (Moreno, Montilla, Villamiel, Corzo, & Olano, 2014; Villamiel, Montilla, Olano, & Corzo, 2014). In rats, Hernandez-Hernandez, Marin-Manzano, Rubio, Moreno, Sanz and Clemente (2012) pointed out a higher resistance of OsLu as compared to GOS to gastrointestinal digestion and absorption in the small intestine, probably due to the β(1→4) linkage between galactose and fructose at the reducing end of the OsLu molecules.

OsLu have been shown to possess bifidogenic effect in *in vitro* pure lactobacilli and bifidobacteria cultures (Cardelle-Cobas, Corzo, Olano, Pelaez, Requena, & Avila, 2011) and in fecal slurries (Cardelle-Cobas et al. 2009; 2012), as well as in experimental animal assays (Marín-Manzano et al. 2013). In addition, in experimental studies with rats, the safety of these oligosaccharides at a concentration of 1.3 g/kg of body weight during 28 days was also demonstrated (Anadón et al. 2013). With similar doses, prebiotic and anti-inflammatory effects were achieved (Hernández-Hernández et al. 2012; Algieri et al. 2014). Additionally, OsLu have also exerted a positive effect on iron absorption in deficient rats (Laparra, Diez-Municio, Herrero and Moreno, 2014).

However, the applicability from a technological point of view of OsLu within a real food is not known so far. In order to have foods containing these ingredients as additives is needed to carry out stability studies in different food matrixes of easy availability and frequent consumption. During thermal processing of foods, different reactions involving prebiotic carbohydrates and/or other food ingredients, namely proteins or amino acids could take place. For this reason, the objective of this work has focused on the characterization of the ingredient containing oligosaccharides derived from lactulose, as well as on the study of the stability of oligosaccharides derived from

lactulose during processing of foods with different composition and pH, namely milk and apple juice.

2. Materials and Methods

2.1. Obtainment of GOS and OsLu

OsLu were obtained at pilot scale by the company Innaves S.A. (Vigo, Spain) following the method described by Anadón et al. (2013). Briefly, OsLu were synthesized using a commercial lactulose preparation (670 g of lactulose per liter; Duphalac, Abbott Biologicals B.V., Olst, The Netherlands) and β-galactosidase from *Aspergillus oryzae* (16 U/mL; Sigma, St. Louis, MO). Enzymatic reactions were carried out at 50ºC and pH 6.5 in an orbital shaker at 300 rpm for 24 h. Afterward, samples were immediately immersed in boiling water for 10 min to inactivate the enzyme. Later, the mixture of oligosaccharides was treated with yeast cells to eliminate monosaccharides, following the method previously described by Sanz et al. (2005) with some changes. Briefly, the oligosaccharide reaction mixture (20% [w/v]) was treated with fresh *Saccharomyces cerevisiae* (1.5% [w/v]; Levital, Paniberica de Levadura S.A., Valladolid, Spain) at 30ºC for 48 h in an orbital shaker (300 rpm) and submitted to vacuum filtration (nylon, 1.2 µm, Millipore, Billerica, MA, USA) to remove the yeast cells. Samples were vacuum dried at 40ºC in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland).

Vivinal®GOS syrup was kindly provided by Borculo Domo (Hanzeplein, The Netherlands) and it had a 73% dry matter (DM), while the composition of carbohydrates was of 60% of GOS, 20% of lactose, 19% of glucose and 1% of galactose.

2.2. Samples

Pasteurized skimmed milk and apple juice were purchased from a local market in Madrid (Spain). Both samples were kept refrigerated until its subsequent manipulation for the assays with the ingredient OsLu.

Sodium phosphate buffer (0.1 M, pH 6.8) and sodium citrate buffer (0.05 M, pH 3.4) were prepared with chemicals of analytical grade (Panreac, Barcelona, Spain). Ultrapure water quality (18.2 MΩ cm) with 1−5 ppb total organic carbon (TOC) and <0.001 EU mL^{-1} pyrogen levels was produced in-house using a laboratory water purification Milli-Q Synthesis A10 system from Millipore (Billerica, MA, USA).

2.3. Thermal treatments of milk and apple juice with/without OsLu added

The thermal treatments were based on the work of de Rafael, Villamiel and Olano (1997), with some modifications. Portions of milk and apple juice (10 mL) with and without OsLu were heated in Pyrex tubes (16 x 1.5 cm) immersed in a thermostatically controlled bath of glycerol at 80 and 100ºC for 10, 20 and 30 min and 80 and 90ºC for 5, 10 and 15 min, respectively. Heating was stopped by rapid cooling of the tubes in an ice-water bath. All assays were done in duplicate. Control samples of phosphate and citrate buffer with OsLu were also heated under identical conditions of milk and apple juice, respectively.

2.4. Storage assays

Milk with and without OsLu samples and thermally treated at 100ºC for 30 min were stored at room temperature (25ºC) for 3 months. Samples were withdrawn in duplicate at 0, 1, 2 and 3 months.

Apple juice with and without OsLu samples and thermally treated at 90ºC for 15 min were stored in refrigeration (4ºC) for 90 days. Samples were taken in duplicate at 0, 15, 30 and 90 days.

2.5. Procedures for the physical-chemical characterization of OsLu

The DM content was gravimetrically determined by drying OsLu and Vivinal®GOS in a conventional oven at 102ºC until constant weight. The °Brix of both types of oligosaccharides were determined using a refractometer (Metter Toledo, 30PX) at 20ºC. The water activity (a_w) measurement of OsLu and Vivinal®GOS was determined at 25ºC using a Novasina a_w Sprint TH-500 (Pfäffikon, Switzerlad) previously calibrated with saturated solutions of different inorganic salts. All assays were performed in duplicate.

The pH of samples was measured using a pH meter MP 225 (Mettler Toledo GmBH, Schwerzenbach, Switzerland) at 20ºC. OsLu or Vivinal®GOS (1 g) were diluted in 10 mL of Milli-Q water.

The mineral composition of OsLu was determined using an ICP-MS Elan 6000 Perkin-Elmer Sciex instrument from the Service Interdepartmental Research (SIdI-UAM) in Madrid. Either a semi-quantitative analysis or a quantitative analysis of the elements of interest using the external calibration method and internal standards to correct instrumental drift were carried out (Zuluaga, Rodriguez, Rivas-Ramirez, de la Fuente, Rufo, & Amils, 2011).

Total nitrogen content was determined by the Kjeldhal method (AOAC, 1990) and protein level was calculated using 6.25 as conversion factor.

In order to evaluate the microbiological quality, OsLu were analyzed for the presence of yeasts and molds, total and sporulated aerobic microorganisms, and enterobacteria. Samples (1.5 g) were placed with 27 mL of peptone water (sterile peptone, 2.55%) in a sterile stomacher bag and then were homogenized into the stomacher for 1 min (230 rpm), filtered and diluted with peptone water for the microbial count. Serial dilutions were performed in triplicate. Yeasts and molds were plated on sulphite cycloserine agar and incubated at 25 ± 1ºC for 5 days. The total and sporulated aerobic bacteria were determined by plating appropriately diluted samples onto plate count agar. The samples were incubated at 30 ± 1ºC for 72 h for total aerobic bacteria and at 37 ± 1ºC for 48 h for sporulated aerobic bacteria after heat treatment of stock dilution at 80ºC for 10 min. For enterobacteria counts, violet red bile dextrose agar was used and incubation was carried out at 30 ± 1ºC for 24 h. All culture media were of Difco (Difco Co., Detroit, MI). All microbial counts were reported as colony forming units per gram (CFU/g). Assays were carried out at least in duplicate.

2.6. Determination of carbohydrates

The carbohydrate composition of samples (OsLu, milk and apple juice with/without OsLu and sodium phosphate and sodium citrate buffers with OsLu) was determined by GC-FID in an Agilent Technologies 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionization detector, using nitrogen as a carrier gas at 1 mL/min.

The trimethylsilyl oximes (TMSO) derivatives were prepared following the method of Ruiz-Matute, Hernandez-Hernandez, Rodriguez-Sanchez, Sanz and Martinez-Castro (2011). OsLu (0.5 g diluted to 10 mL with Milli-Q water) or milk, apple juice or buffer with/without OsLu samples (0.5 mL) were diluted to 10 mL with

methanol. A volume of 1 mL of supernatant was added to 400 µL of phenyl-β-D-glucoside (internal standard). The mixture was vacuum dried at 40ºC in a rotary evaporator. Sugar oximes were formed by adding 250 µL hydroxylamine chloride (2.5%) in pyridine and heating the mixture at 70ºC for 30 min. Subsequently, the oximes were silylated with hexamethyldisilazane (250 µL) and trifluoroacetic acid (25 µL) at 50ºC for 30 min. Reaction mixtures were centrifuged at 10000 rpm for 2 min. Supernatants were injected in the GC or stored at 4ºC prior to analysis. Injections were made in the split mode (1:20).

The TMSO were separated using a fused-silica capillary column (30 m x 0.32 mm i.d. x 0.5 µm film thickness) SPBTM-17, bonded, crosslinked phase (50% diphenyl-50% dimethylsiloxane) (Supelco, Bellefonte, PA, USA). In the case of milk and phosphate buffer samples the oven initial temperature was 200°C, increased at a rate of 4°C/min to 230°C, increased 1°C/min to 250°C, increased at 2ºC/min to 290ºC, and held for 62 min. In the case of apple juice and citrate buffer samples the oven initial temperature was 140°C, increased at a rate of 4°C/min to 155°C, increased at 10°C/min to 230°C, 1ºC/min to 250ºC, 2ºC/min to 290ºC and was held for 62 min. The injector and detector temperatures were 280 and 290°C, respectively.

Data acquisition and integration were performed using Agilent ChemStation software (Wilmington, DE, USA). Quantitative data for carbohydrates were calculated from FID peak areas relative to phenyl-β-D-glucoside. OsLu data was expressed as percentage of carbohydrates, while milk and apple juice with OsLu added samples data was expressed as mg/100 mL of mixture or product.

2.7. Determination of Maillard reaction indicators

Determination of furosine in milk with and without OsLu was carried out by ion-pair RP-HPLC following the method of Ruiz-Matute, Corzo-Martinez, Montilla, Olano, Copovi and Corzo (2012). Before analysis, samples (1 mL) were hydrolyzed with 3 mL of 10.6 N HCl under inert conditions at 110ºC for 24 h in Pyrex tubes. The hydrolyzate was filtered through Whatman Nº 40 filter paper and 0.5 mL of filtrate was applied to a previously activated (methanol and water) Sep-Pak C_{18} cartridge (Waters). Furosine was eluted with 3 mL of 3 N HCl and 50 µL was injected into the chromatograph. RP-HPLC analysis of furosine was carried out in a C_8 column (250 cm x 4.6 mm inside diameter) (Alltech furosine-dedicated, Nicolasville, KY) maintained at 37ºC using a linear binary gradient at a flow rate of 1.2 mL/min. Mobile phase was constituted by solvent A, 0.4% acetic acid, and solvent B, 0.34% KCl in phase A. The elution program was as follows: 100% A from 0 to 12.5 min, 50% A from 19.5 to 24 min, and 100% A from 24 to 32 min. Detection was done using a variable wavelength UV detector at 280 nm (LDC Analytical, SM 4000, Salem, NH). Acquisition and processing of data were achieved with System Gold (Beckman) software. Quantification was carried out by the external standard method, using a commercial standard of pure furosine (Neosystem Laboratories, Strasbourg, France). All analyses were done in quadruplicate, and the data are the mean values expressed mg/100 g of protein.

Final steps of MR in samples of milk and apple juice with/without OsLu were determined by measuring the absorbance at 420 nm using a spectrophotometer UV-Vis (Power Wave XS Microplate, BIO-TEK) and the KC Junior Data Reduction software. In the case of milk, 0.5 mL were added to 0.25 mL of trichloroacetic acid/water (40:60). The mixture was centrifuged for 10 min at 9600*g* and the supernatant was filtered (0.45 µm) (Guerra-Hernandez, Gomez, Garcia-Villanova, Sanchez, & Gomez, 2002). In the case of apple juice, samples were centrifuged at 10ºC for 10 min at 9600*g* (Ting, &

Rouseff, 1986). The absorbance at 420 nm was measured in both cases in the supernatant obtained after centrifugation.

2.8. Sensorial analysis

The sensorial analysis was carried out by a taste panel of 20 semi-trained judges. A triangle test procedure was followed to compare samples of apple juice with and without OsLu. Panelists were presented with two groups of three samples each, distributed so that in each group two samples were the same and another was different in a certain order (Watts, Ylimaki, Jeffery, & Elías, 1992). Panelists were asked to identify the odd sample. A hedonic test procedure was followed to compare apple juice with OsLu added and apple juice or apple juice with Vivinal®GOS added samples. The panelists were asked to indicate their preference for each sample. A balanced 8-point hedonic rating was employed, where one denoted "like very much" and eight indicated "dislike very much" (Sancho, Bota, & de Castro, 2002).

2.9. Statistical analysis

The comparisons of means using analysis of variance (ANOVA) were made using the statistical software Microsoft Excel of Microsoft Office 2010. The differences were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Characterization of OsLu

Table 1 shows different physicochemical parameters and chemical composition of the OsLu ingredient. In agreement with the °Brix, DM was very high, due to the high

concentration of carbohydrates, as corresponds to a syrup formula. The value of a_w (0.58) indicated the potential stability of this ingredient against the chemical, enzymatic and microbiological impairment, since at values close to 0.5 these modifications are slowed. Moreover, the low pH value (3.01) and the analysis of total aerobic bacteria, enterobacteria, yeast and molds, aerobic and anaerobic sporulated counts ($\leq 4.3 \times 10^2$) also guarantee the microbial stability of OsLu during the storage.

Although in low amount (0.46%), proteins were also present in the ingredient, probably derived from the enzymes used for its obtainment and yeast purification. The content of salts was very scarce as well. The main detected elements were K (905.2 μg/g), Na (76.3 μg/g), Si (59.1μg/g), Ca (21.0μg/g) and Mg (9.5 μg/g) and resulted from the isomerization and transgalactosylation processes used to form lactulose and its derivatives (OsLu), respectively.

The carbohydrate composition of OsLu was also determined. Figure 1 illustrates the chromatographic profile of the TMS oxime derivatives corresponding to galactose, lactulose, lactose and OsLu disaccharides, trisaccharides and tetrasaccharides. As observed in Table 1, the percentage of potential prebiotic carbohydrates (lactulose, OsLu disaccharides, trisaccharides and tetrasaccharides) was higher in the OsLu ingredient (67%) as compared to Vivinal®GOS (59%) (Hernandez-Hernandez, Sanz, Kolida, Rastall, & Moreno, 2011), trisaccharides being the most abundant fraction (22.3%). The content of monosaccharides and lactose, non-prebiotic carbohydrates that increase the caloric power of the ingredient, was lower in the former and, moreover, no presence of glucose was detected; thus, with the same dose, a higher beneficial effect might be expected in the case of OsLu. Therefore, this new prebiotic ingredient could be used for a wide range of population, including diabetics and lactose intolerants.

3.2. Effect of processing of milk

After the characterization of OsLu, an addition of this ingredient was done in milk and apple juice before thermal treatments (80-100°C for 5-30 min). According to the works of Walton, van den Heuvel, Kosters, Rastall, Tuohy, & Gibson (2012) and Whisner et al. (2013) a dose within the range 5-10 g/day of Vivinal®GOS is needed to observe a significant bifidogenic effect. On the basis of these previous papers, we decided to add 5 g of OsLu per 100 mL of food (3.3 g of prebiotic carbohydrates/100 mL), taking into account a daily consumption of 300 mL of milk or juice. Sodium phosphate buffer (pH 6.8) with added OsLu was used as control to isolate the matrix effect. Once the samples were processed, an assessment of changes in the carbohydrate fraction was carried out. The main changes were observed in samples subjected to the most severe conditions (100°C for 30 min) and hardly any change in the carbohydrates with polymerization degree ≥3 was detected. Figure 2 depicts, as an example, the chromatogram of milk with and without OsLu treated at 100°C for 30 min. As expected, the observed peaks corresponded mainly to the ingredient (galactose, lactulose and OsLu disaccharides, trisaccharides and tetrasaccharides) and milk (glucose, *myo*-inositol, N-acetyl-glucosamine, N-acetyl-galactosamine and lactose). Table 2 shows the quantitative data of all carbohydrates analyzed in samples of milk before and after thermal treatment with and without addition of OsLu, as well as those of the corresponding phosphate buffer. The levels of *myo*-inositol, N-acetyl-glucosamine, N-acetyl-galactosamine and lactose kept constant in the heated milk samples, in agreement with the data of Troyano, Villamiel, Olano, Sanz & Martinez-Castro (1996). The amount of galactose and lactulose increased significantly with the intensity of thermal treatment in milk without OsLu, since it is well known that during the heating, lactose is isomerized to lactulose which is subsequently degraded to galactose and saccharinic

293 acids (Troyano et al. 1996). In the case of milk with OsLu, the significant increase of
294 lactulose content was accompanied by a notable but not significant increase in the
295 concentration of galactose, probably due to a decrease in the pH of milk after the
296 addition of OsLu. As it is known, the rate of this reaction is higher with high values of
297 pH in the medium (Berg, 1993; Moreno, Villamiel, & Olano, 2003). In sodium
298 phosphate buffer, the significant degradation of lactulose to galactose was also observed
299 but with a significant decrease in lactulose content, in contrast to the result of milk. In
300 this case, the scarce presence of lactose avoided the formation of lactulose, in spite of
301 the favorable thermal conditions for isomerization of carbohydrates.

302 Regarding OsLu fraction (OsLu disaccharides, trisaccharides and
303 tetrasaccharides), no modification was observed, if any, after the thermal treatment of
304 milk with OsLu, indicating the great stability of this ingredient in this matrix under the
305 thermal treatments assayed. In the sodium phosphate buffer, however, significant
306 decrease and increase in tetrasaccharides and disaccharides, respectively, and no
307 modification of trisaccharide content were detected. This result could be ascribed to the
308 partial hydrolysis of tetrasaccharides to form trisaccharides which could be also
309 transformed into disaccharides provoke the galactose release and increasing the
310 galactose content observed. The different behavior of this fraction of carbohydrates in
311 milk and in sodium buffer phosphate indicates a certain matrix protective effect on its
312 degradation during the heating of milk.

313 During processing of milk other changes that can be produced in the
314 carbohydrate fraction, namely in reducing sugars, is its interaction with proteins via the
315 Maillard reaction (MR). In this sense, we evaluated the formation of furosine, as
316 indicator of the initial steps of this reaction, in milk with and without OsLu subjected to
317 80 and 100°C for 10-30 min (Table 3). Furosine was detected in all samples, and

318 increased with the severity of heating, temperature being the most influencing factor in
319 the reaction (de Rafael et al. 1997). The observed values of furosine were within the
320 range found previously for milk samples heated under similar conditions (Villamiel,
321 Corzo, Martinez-Castro, & Olano, 1996).

322 Comparing both types of samples, with the exception of milks treated at 80 and
323 100°C for 30 min, a significant increase in the amount of furosine was found in samples
324 with OsLu, probably due to the high concentration of galactose in the ingredient (Table
325 1). Galactose is a very reactive carbohydrate that can react with the amino groups of
326 proteins giving rise to the corresponding Amadori compound during the initial stages of
327 MR, which after acid hydrolysis forms furosine (Evangelisti, Calcagno, Nardi & Zunin,
328 1999). After 30 min of heating no significant differences were detected in the content of
329 furosine due to the fact that the rates of formation and degradation of Amadori
330 compound could start to be equilibrated (Erbersdobler & Somoza, 2007). The final steps
331 of MR were also studied (results not shown) and hardly any change in the absorbance at
332 420 nm was detected and all the values were within the range 0.052-0.115.

333 On the other hand, the formation of highest concentration of furosine in the
334 samples with OsLu does not contitute a drawback for the application of this ingredient
335 as prebiotic, since, during the initial steps of MR, galactose can be isomerized to
336 tagatose forming the Amadori compound tagatosyl-lysine, complex that could resist the
337 conditions of digestion and reach the gut to exert its positive effect, as demonstrated
338 Corzo-Martinez, Hernandez-Hernandez, Villamiel, Rastall, & Moreno (2013).

339 The modifications of composition were also evaluated in milk samples with and
340 without OsLu heated at 100°C for 30 min and stored at room temperature for three
341 months. Change was detected neither in the carbohydrate fraction nor in the formation

342 of furosine (data not shown), indicating the stability of this ingredient to the storage
343 conditions.

344 *3.3. Effect of processing of apple juice*

345 A similar study was also carried out in the case of apple juice with addition of
346 OsLu ingredient after the treatment at 80-90°C for 5-15 min. In this case sodium citrate
347 buffer (pH 3.4) was used as control. Figure 3 illustrates, as example, the
348 chromatographic profile of the TMS oxime derivatives of the corresponding
349 carbohydrates. Sorbitol, *myo*-inositol, fructose, glucose and sucrose were derived from
350 the apple juice and galactose, lactulose and OsLu disaccharides, trisaccharides and
351 tetrasacharides from the OsLu ingredient. As can be seen in Table 4, the most striking
352 feature was the decrease in the content of sucrose, due to its hydrolysis into glucose and
353 fructose in acid medium (Ibarz, Garza, Garvin, & Pagan, 2011). The concentration of
354 lactulose and all its derivatives of higher DP, did not suffer modifications as
355 consequence of the processing of apple juice and sodium citrate buffer, probably due to
356 the low pH value. These results are in agreement with those previously reported for
357 Vivinal®GOS heated at 100°C in a model system of pH 3 (Van Leusen et al. 2014).
358 Klewicki (2007) studied the stability of apple beverages (pH 3.4) against treatments at
359 95°C, 3 s followed by 84°C, 20 min and a slight decrease of 1-2% was observed in the
360 content of GOS. This high stability is probably ascribed to the presence of β-type
361 linkages between galactose and glucose (Sangwan, Tomar, Singh, Singh, & Ali, 2011).
362 However, FOS are more sensible to the hydrolysis under these conditions due to the
363 type of glycosidic bond at the terminal end between glucose and fructose. Thus,
364 Matusek, Meresz, Le, & Orsi (2011) found a decrease of 80% in FOS amount in heated
365 juices.

366 To evaluate the evolution of non-enzymatic browning, the absorbance at 420 nm
367 was determination in all samples after thermal treatment and all the values were within
368 the range 0.052-0.434, indicating the scarce advance of this reaction.

369 The stability of OsLu during storage was also studied and no change in the
370 carbohydrate fraction was detected in samples stored at 4°C during 90 days (results not
371 shown).

372 *3.4. Sensory evaluation*

373 The sensory evaluation of the samples of apple juice with OsLu addition was
374 carried out to obtain preliminary information on consumer's preference and product
375 acceptance.

376 In the triangle test, each panelist was presented with two trios in which one of
377 the samples corresponded to apple juice or apple juice with OsLu. Only the 35% of
378 panelists could distinguish the odd sample in both trios, the 45% found the different
379 sample in one of the trios and the 20% did not get right any of the trios.

380 With respect to hedonic evaluation, the mean overall liking marks of the
381 evaluated samples are shown in Table 5. As observed, significant differences between
382 apple juice and apple juice plus OsLu ingredient were found, with preference of the
383 former by the panelists. The best score of apple juice with OsLu was obtained in the
384 case of the comparison with apple juice with Vivinal®GOS and no significant
385 differences were detected between both types of samples with qualifications close to
386 "like moderately".

387

388 **4. Conclusion**

According to these results, it can be inferred that the oligosaccharide fraction present in the OsLu ingredient is stable during the processing and storage conditions in a variety of processed foods with pH in the range 3.4-6.8, as juice and milk. This mixture can be safely used even in special foods destined to diabetics and people with intolerance to lactose. The high presence of prebiotic carbohydrates in the ingredient (67%) facilitates its management since, as compared to other commercial preparations, lower amounts of product might be needed to achieve similar beneficial effects. In addition, the sensorial properties of apple juice with OsLu were acceptable and similar to those of apple juice with addition of a commercial mixture such as Vivinal®GOS. The results here obtained afford important information to be considered in the industrial elaboration of functional foods with prebiotic ingredients.

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559 **Figure caption**

560 **Figure 1.** GC-FID profile of TMSO derivatives of carbohydrates present in OsLu
561 ingredient. Peaks: 1: Galactose$_1$, 2: Galactose$_2$, I.S.: Internal standard (phenyl-β-D-
562 glucoside), 3: Lactulose$_1$, 4: Lactose$_1$+Lactulose$_2$, 5: Lactose$_2$, 6: Other disaccharides, 7:
563 Trisaccharides and 8: Tetrasaccharides.

564 **Figure 2.** GC-FID profile of TMSO derivatives of carbohydrates present in (A) milk
565 treated at 100°C for 30 min. (I) 1: Galactose$_1$, 2: Glucose$_1$, 3: Galactose$_2$+Glucose$_2$, 4:
566 *myo*-inositol, 5: *N*-acetyl-glucosamine$_1$, 6: *N*-acetyl-glucosamine$_2$, 7: *N*-acetyl-
567 galactosamine$_1$, 8: *N*-acetyl-galactosamine$_2$; and full chromatogram: I.S.: Internal
568 standard (phenyl-β-D_glucoside), 9: Lactulose$_1$, 10: Lactulose$_2$+Lactose$_1$ and 11:
569 Lactose$_2$. (B) Milk with OsLu treated at 100°C for 30 min. (II) 2: Glucose$_1$, 4: *myo*-
570 inositol, 5: *N*-acetyl-glucosamine$_1$, 6: *N*-acetyl-glucosamine$_2$, 7: *N*-acetyl-
571 galactosamine$_1$, 8: *N*-acetyl-galactosamine$_2$; and full chromatogram: 1: Galactose$_1$, 3:
572 Galactose$_2$+Glucose$_2$, I.S.: Internal standard (phenyl-β-glucoside), 9: Lactulose$_1$, 10:
573 Lactose$_1$+Lactulose$_2$, 11: Lactose$_2$, 12: Other disaccharides, 13: Trisaccharides and 14:
574 Tetrasaccharides.

575 **Figure 3.** GC-FID profile of TMSO derivatives of carbohydrates present in (A) apple
576 juice treated at 90°C for 15 min. Peaks: 1: Sorbitol, 2: Fructose$_1$, 3: Fructose$_2$, 4:
577 Galactose$_1$, 5: Glucose$_1$, 6: Galactose$_2$+Glucose$_2$, 7: *myo*-inositol, I.S.: Internal standard
578 (phenyl-β-glucoside) and 8: Sucrose. (B) Apple juice with OsLu treated at 90°C for 15
579 min. Peaks: 1: Sorbitol, 2: Fructose$_1$, 3: Fructose$_2$, 4: Galactose$_1$, 5: Glucose$_1$, 6:
580 Galactose$_2$+Glucose$_2$, 7: *myo*-inositol, I.S.: Internal standard (phenyl-β-glucoside), 8:
581 Sucrose, 9: Lactulose$_1$, 10: Lactulose$_2$, 11: Other disaccharides, 12: Trisaccharides and
582 13: Tetrasaccharides.

Figure 1.

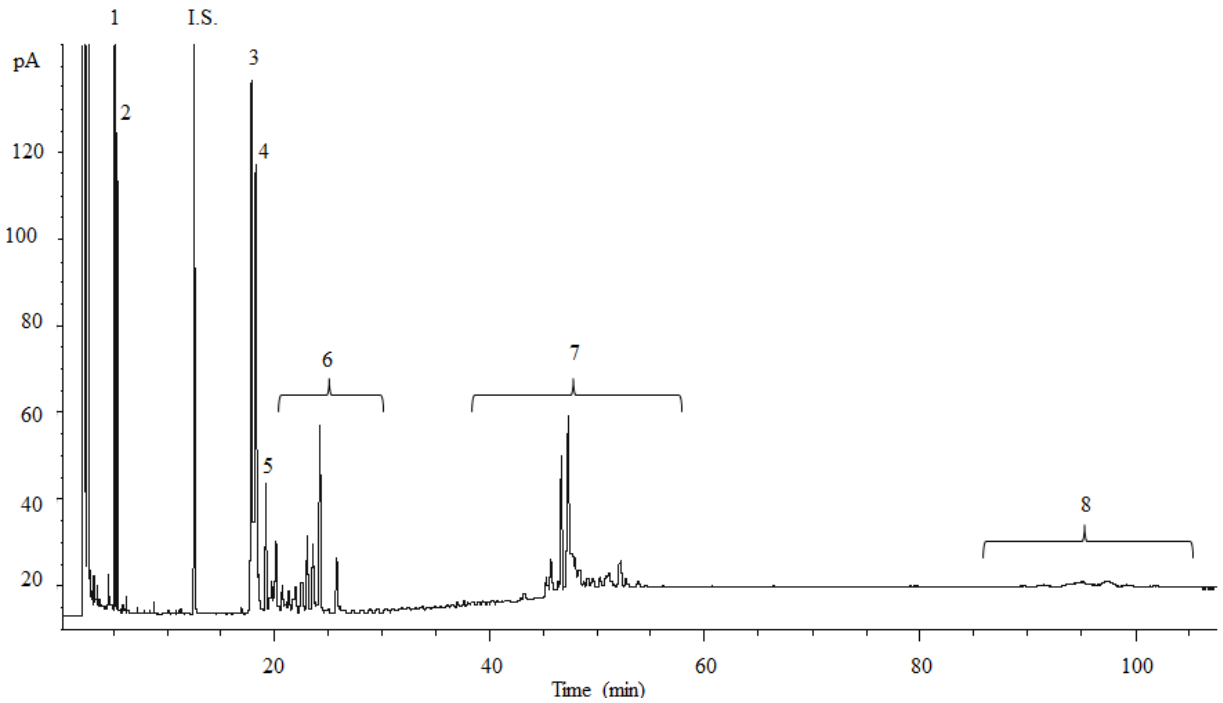

Figure 2.

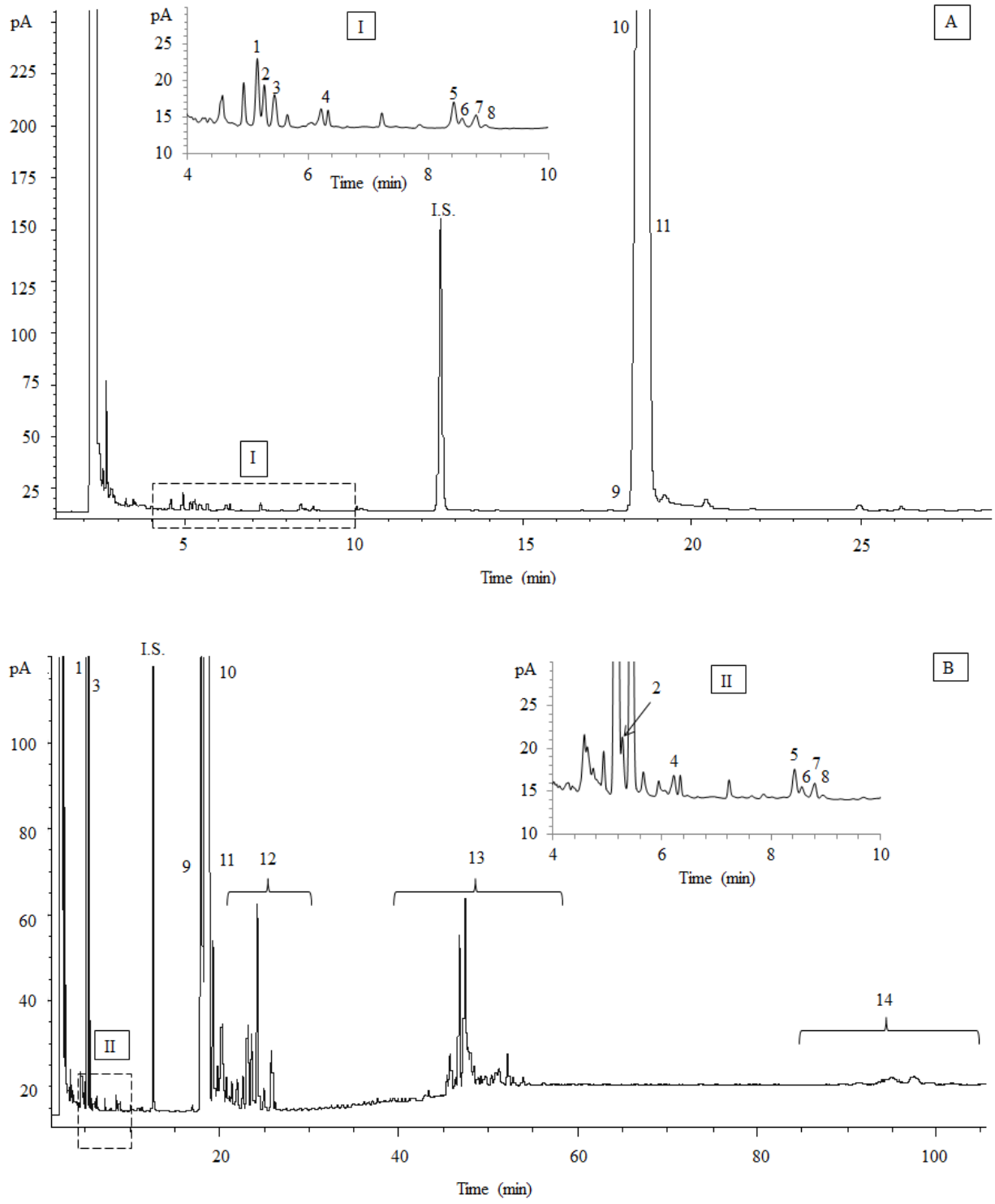

Figure 3.

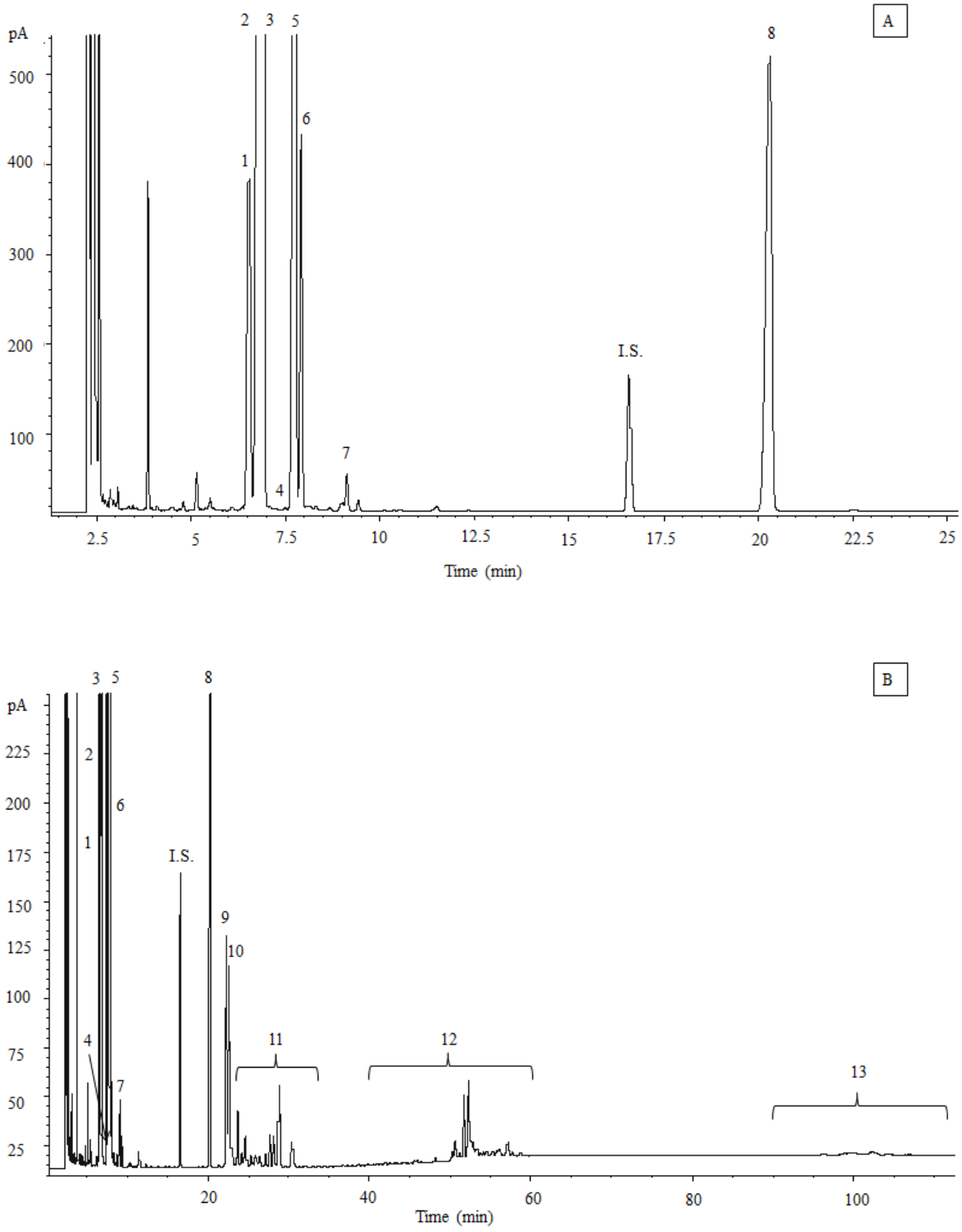
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Table 1. Physicochemical parameters and chemical composition of the OsLu ingredient.

Parameter	**Value**
Dry Matter (%)	80.97
^{o}Brix	83.7
a_w	0.58
pH	3.01
Composition (%)*	**Value**
Galactose	10.95
Lactose	3.31
Lactulose	19.58
OsLu Disaccharides	18.33
OsLu Trisaccharides	22.30
OsLu Tetrasaccharides	6.38
Salts	0.13

**These values are per 100 g of ingredient.*

Table 2. Content of carbohydrates in mg/100 mL in samples of milk before and after thermal treatment (100ºC 30 min) with and without addition of OsLu, as well as those of the corresponding phosphate buffer (*n*=4). The values are media ± standard desviation.

	Galactose	**Glucose**	**N-acetyl-glucosamine**	**N-acetyl-galactosamine**	***myo*-inositol**	**Lactulose**	**Lactose**	**OsLu Disaccharides**	**OsLu Trisaccharides**	**OsLu Tetrasaccharides**
Milk 25ºC	7.57 [a] (± 0.31)	8.42 [a] (± 0.73)	11.99 [a] (± 0.65)	6.95 [a] (± 0.54)	3.38 [a] (± 0.07)	2.31 [a] (± 1.28)	5453.39 [a] (± 186.20)			
Milk 100ºC 30min	16.22 [b] (± 3.15)	8.10 [a] (± 0.59)	11.59 [a] (± 0.64)	6.44 [a] (± 0.12)	3.47 [a] (± 0.22)	85.47 [b] (± 7.52)	5393.55 [a] (± 411.32)			
Milk with OsLu 25ºC	490.83 [a] (± 25.09)	8.74 [a] (± 1.38)	11.24 [a] (± 0.56)	5.96 [a] (± 0.57)	3.56 [a] (± 0.21)	816.08 [a] (± 53.52)	5040.40 [a] (± 305.05)	850.26 [a] (± 61.06)	919.25 [a] (± 61.93)	281.20 [a] (± 27.25)
Milk with OsLu 100ºC 30min	527.60 [a] (± 30.77)	11.53 [b] (± 1.48)	11.31 [a] (± 0.49)	6.71 [a] (± 0.28)	3.77 [a] (± 0.22)	926.38 [b] (± 52.60)	5246.48 [a] (± 157.28)	926.59 [a] (± 46.05)	974.80 [a] (± 47.21)	290.03 [a] (± 20.28)
Phosphate buffer pH 6.8 with OsLu 25ºC	517.98 [a] (± 7.43)					915.37 [a] (± 9.15)		873.74 [a] (± 21.80)	972.69 [a] (± 48.94)	287.07 [a] (± 13.26)
Phosphate buffer pH 6.8 with OsLu 100ºC 30min	551.39 [b] (± 8.61)					853.42 [b] (± 15.31)		912.16 [b] (± 7.64)	966.92 [a] (± 62.98)	257.96 [b] (± 8.73)

**Different letters indicate significant differences (P<0.05).*

Table 3. Evaluation of the formation of furosine in mg/100 g protein, as indicator of the initial steps of the Maillard reaction, in milk with and without OsLu subjected to 80 and 100°C for 10-30 min (*n*=4). The values are media ± standard desviation.

Temperature (ºC)	Time (min)	Furosine (mg/100 g protein)	
		Milk	Milk + OsLu
80	**10**	18.89 [a] (±1.36)	26.24 [b] (±1.92)
	20	28.48 [a] (±5.21)	40.63 [b] (±4.98)
	30	40.64 [a] (±2.55)	48.63 [a] (±7.78)
100	**10**	109.38 [a] (±5.35)	136.36 [b] (±3.18)
	20	157.33 [a] (±2.68)	192.56 [b] (±9.19)
	30	180.69 [a] (±16.28)	216.52 [a] (±18.49)

**Different letters indicate significant differences (P<0.05).*

Table 4. Content of carbohydrates in mg/100 mL in samples of apple juice before and after thermal treatment (90ºC 15 min) with and without addition of OsLu, as well as those of the corresponding citrate buffer (*n*=4). The values are media ± standard desviation.

Samples	Fructose	Galactose	Glucose	Sorbitol	*myo*-inositol	Sucrose	Lactulose	OsLu Disaccharides	OsLu Trisaccharides	OsLu Tetrasaccharides
Apple juice 25ºC	6570.47 [a] (± 71.03)	13.50 [a] (± 0.78)	2896.63 [a] (± 30.05)	563.38 [a] (± 5.98)	15.30 [a] (± 0.66)	2080.74 [a] (± 35.33)				
Apple juice 90ºC 15 min	6623.08 [a] (± 127.14)	13.66 [a] (± 0.22)	3005.75 [b] (± 60.26)	560.08 [a] (± 9.68)	15.74 [a] (± 0.66)	1866.45 [b] (± 45.18)				
Apple juice with OsLu 25ºC	6497.96 [a] (± 76.23)	532.48 [a] (± 5.36)	3051.01 [a] (± 31.32)	533.64 [a] (± 6.64)	15.52 [a] (± 0.45)	1380.93 [a] (± 12.38)	893.42 [a] (± 5.43)	965.97 [a] (± 23.97)	973.10 [a] (± 40.71)	286.82 [a] (± 11.34)
Apple juice with OsLu 90ºC 15 min	6761.77 [b] (± 126.42)	533.49 [a] (± 10.26)	3286.94 [b] (± 60.01)	540.13 [a] (± 10.57)	15.59 [a] (± 0.44)	961.00 [b] (±16.14)	901.30 [a] (± 14.78)	959.87 [a] (± 19.10)	965.63 [a] (± 9.81)	285.97 [a] (± 11.21)
Citrate buffer with OsLu 25ºC		529.165 [a] (± 7.78)	1.76 [a] (± 0.09)				946.51 [a] (± 22.09)	970.94 [a] (± 10.98)	986.69 [a] (± 28.82)	279.25 [a] (± 9.12)
Citrate buffer with OsLu 90ºC 15 min		515.49 [a] (± 8.59)	1.81 [a] (± 0.18)				946.47 [a] (± 21.97)	974.03 [a] (± 32.13)	993.36 [a] (± 24.01)	268.41 [a] (± 11.84)

**Different letters indicate significant differences (P<0.05).*

Table 5. Scores by the panelists in the hedonic evaluation (*n*=20) (scale 1-8; 1 “like very much”, 8 “dislike very much”).

Samples	Scores	
Apple juice	2.55 [a] (± 0.6)	
Apple juice with OsLu	4 [b] (± 0.97)	3.85 [a] (± 1.09)
Apple juice with Vivinal ® GOS		3.15 [a] (± 1.09)

**Different letters indicate significant differences (P<0.05).*

Figure 1S. Supplemental information Lopez-Sanz *et al.*

GC-FID profile of TMSO derivatives of carbohydrates present in OsLu ingredient. Peaks: 1: Galactose1, 2: Galactose2, I.S.: Internal standard (phenyl-β-D-glucoside), 3: Lactulose1, 4: Lactose1+Lactulose2, 5: Lactose2, 6: Other disaccharides, 7: Trisaccharides and 8: Tetrasaccharides.

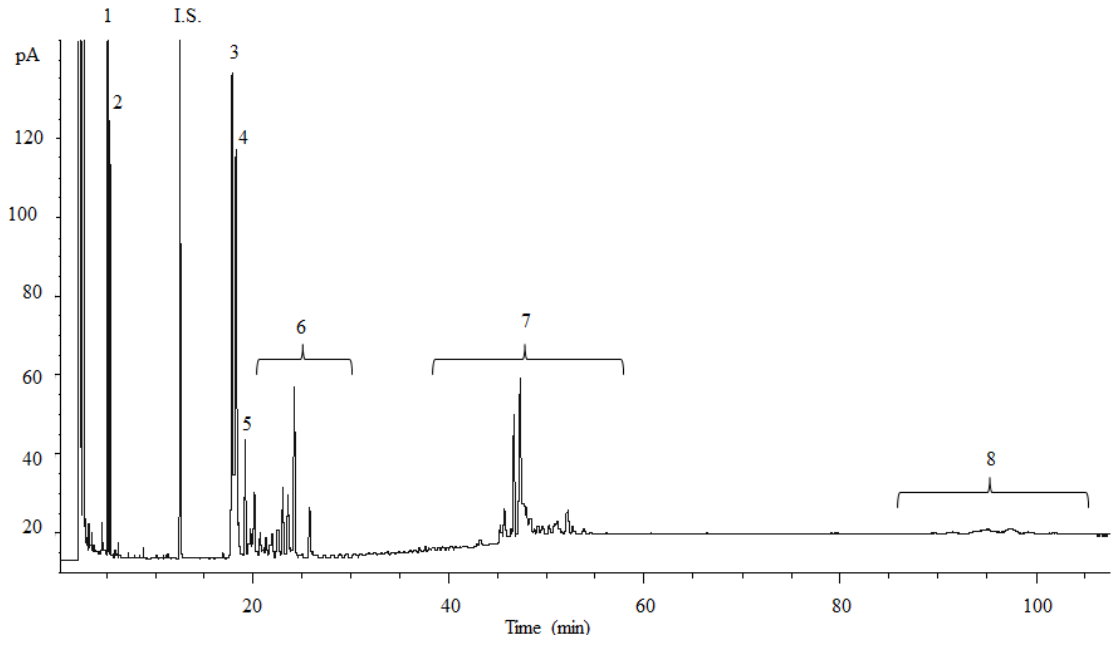

Table 1S. Supplemental information Lopez-Sanz *et al.*

Scores by the panelists in the hedonic evaluation (*n*=20) (scale 1-8; 1 “like very much”, 8 “dislike very much”).

Samples	Scores	
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Apple juice with OsLu	4 [b] (± 0.97)	3.85 [a] (± 1.09)
Apple juice with Vivinal ® GOS		3.15 [a] (± 1.09)

**Different letters indicate significant differences (P<0.05).*

Highlights (3-5, max 85 caracteres)

* Oligosaccharides from lactulose (OsLu) have high content of prebiotic compounds

* Oligosaccharides from lactulose are stable during processing and storage

* OsLu are constituted by high content of prebiotic carbohydrates mainly trisaccharides

* Organoleptic analysis of apple juice with OsLu shown adequate value

*OsLu can be used in the elaboration of foodstuffs at different pH