Effect of glycation of bovine β-lactoglobulin with galactooligosaccharides on the growth of human faecal bacteria

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Abstract

The *in vitro* fermentation selectivity of purified galactooligosaccharides (GOS) after their conjugation with bovine β-lactoglobulin (β-LG) via the Maillard reaction and a subsequent simulated gastrointestinal digestion was evaluated. Changes in human faecal bacterial populations, lactic acid and short-chain fatty acids after 10 and 24h of fermentation of the digested β-LG:GOS conjugates revealed that this mixture of glycated peptides had a similar bifidogenic activity to the unconjugated GOS. These findings could open up new applications of Maillard reaction products in the functional foods field.

1. Introduction.

Protein glycation is frequently used to improve protein functionality and, consequently, to obtain new functional food ingredients with improved biological and technological properties (Oliver, Melton, & Stanley, 2006). Previously, the glycation of bovine β-lactoglobulin (β-LG) with galactooligosaccharides (GOS) with polymerization degrees from 2 to 7 was evaluated (Sanz, Corzo-Martinez, Rastall, Olano, & Moreno, 2007). *In vitro* simulated gastrointestinal digestion revealed the complete hydrolysis of unglycated and glycated β-LG and the formation of stable glycated peptides which were characterised by LC−ESI-MS/MS (Moreno, Quintanilla-Lopez, Lebron-Aguilar, Olano, & Sanz, 2008).

Some *in vitro* studies and *in vivo* experiments in rats have shown that non-digested Amadori compounds, the first stable intermediates in the Maillard reaction, could reach the colon where they are fermented by microorganisms (Erbersdobler, & Faist, 2001; Faist, & Erbersdobler, 2001; Finot, 2005). Therefore, the conjugation between prebiotic carbohydrates and food proteins could potentially allow carbohydrates to reach the distal parts of the colon, where many chronic gut disorders originate (Gibson, Probert, Loo, Rastall, & Roberfroid, 2004). However, it is vital to confirm that prebiotics maintain their fermentation selectivity when they are linked to peptides. There is currently very little information on fermentation selectivity of Maillard reaction products. A non-specific increase in the anaerobic bacteria was reported after the fermentation of melanoidins, final products of Maillard reaction, produced from an aqueous glucose–lysine model system (Ames, Wynne, Hofmann, Plos, & Gibson, 1999). Also, bread crust melanoidins stimulated

growth of bifidobacteria (Borrelli, & Fogliano, 2005). In contrast, other studies showed that bovine serum albumin (BSA) glycated with glucose promoted the growth of detrimental species of bacteria (sulphate-reducing bacteria and clostridia) instead of that of bifidobacteria and lactobacilli compared to native BSA (Tuohy et al., 2006). Moreover, pure culture studies revealed that Maillard reaction products derived from roasted cocoa bean reduced the growth of *E. coli* spp., *Enterobacter cloaceae* and bifidobacteria (Summa et al., 2008).

Nevertheless, none of the aforementioned studies evaluated the effect of a protein glycated with prebiotic carbohydrates on the growth of human gut bacteria after an *in vitro* gastrointestinal (GI) digestion. Huebner, Wehling, Parkhurst, & Hutkins, (2008) studied the effect of Maillard reaction conditions on the prebiotic activity of different commercial fructooligosaccharides (FOS) conjugated with glycine using pure culture assays. However, these prebiotics were not previously fractionated and the formation of the MRPs could be attributed to the presence of minor reducing sugars such as glucose and fructose.

Therefore, the objective of this study was to evaluate the fermentation selectivity of simulated GI-digested β-LG glycated with purified GOS (β-LG:GOS) using small-scale pH-controlled fecal batch cultures and to compare with previously documented activity of GOS (Palframan, Gibson, & Rastall, 2003; Tzortzis, Goulas, Baillon, Gibson, & Rastall, 2004; Vulevic, Rastall, & Gibson, 2004) in order to determine the effect of glycation.

2. Materials and Methods.

2.1. Glycation of β-lactoglobulin with GOS.

The glycation was carried out following the method of Sanz et al. (2007) with some modifications. Aliquots of β-LG (mixture of A and B variants, Sigma-Aldrich, (St. Louis, MO, US)) and Vivinal-GOS® (kindly provided by Friesland Foods Domo, Zwolle, The Netherlands), previously purified by SEC to remove mono- and disaccharides (Hernández, Ruiz-Matute, Olano, Moreno, & Sanz, 2009), were mixed (1:1, w:w) in 0.1 M sodium phosphate buffer (pH 7.0), freeze dried and kept under vacuum in a desiccator (40 ºC, a_w: 0.44, 16 days). Unglycated β-LG without GOS was dissolved in 0.1 M sodium phosphate buffer (pH 7.0), freeze dried and incubated under the same conditions to be used as control.

2.2. Determination of conjugated GOS.

After the storage of β-LG and GOS, the unconjugated GOS fraction was removed by ultrafiltration using a diafiltration unit Model 8400 (Millipore Corp., Bedford, MA, USA) with a molecular weight cut-off of 10 kDa; the retentate was analyzed by ESI-MS to confirm the absence of unconjugated GOS. The permeate was analysed by gas chromatography-mass spectrometry (GC-MS) using a two-step derivatization (oximation and trimethylsilylation) as previously reported by Hernandez et al. (2009) to determine the percentage of unconjugated GOS (tri- and tetrasaccharide fractions). Thus, the quantity of conjugated GOS was calculated by using this equation:

GOS conjugated = Total GOS - GOS unconjugated

where total GOS is the amount of tri- and tetrasaccharide fractions, quantified by GC, used at the initial of the conjugation process.

92 *2.3 Gastrointestinal digestion and characterization of β-LG:GOS conjugates.*

93 β-LG:GOS conjugates and the unglycated β-LG were digested following the
94 simplified method proposed by Moreno, Mellon, Wickham, Bottrill, & Mills (2005) and
95 characterized as indicated by Sanz et al. (2007) and Moreno et al. (2008).

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97 *2.4 In vitro fermentations and analyses.*

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99 Faecal samples were obtained from three healthy donors (aged 25-30 years old).
100 Samples were diluted (1:10, w:v) with phosphate buffer (0.1 M, pH 7.4) and homogenised
101 in a stomacher for 2 min at normal speed. Small scale pH controlled batch cultures (pH
102 between 6.7 and 6.9) were used for the incubation of 50 mg of digested β-LG, β-LG:GOS
103 and free GOS (positive control) with 0.05 mL of the faecal slurry at 37 ºC dissolved in 5
104 mL of autoclaved nutrient basal medium. In addition, a control without substrate was
105 carried out (negative control). Samples were taken after 0, 10 and 24 h of incubation.
106 Fluorescent in situ hybridization (FISH) was carried out following the method proposed by
107 Martin-Pelaez et al. (2008) using 16S rRNA-targeted oligonucleotide probes labelled with
108 Cy3. Probes (Sigma) were as follows: Bif164 specific for *Bifidobacterium* (Langendijk et
109 al., 1995), Bac303 specific to *Bacteroides* (Manz, Amann, Ludwig, Vancanneyt, &
110 Schleifer, 1996), Chis150 for the *Clostridium histolyticum* group clusters I, II (Franks et al.,
111 1998), Erec482 for the *Clostridium coccoides – Eubacterium rectal* group (Franks et al.,
112 1998), Lab158 for *Lactobacillus-Enterococcus* group (Harmsen, Elfferich, Schut, &
113 Welling, 1999) and Ato291 for the *Atopobium* cluster (Harmsen et al., 1999). For total
114 counts the nucleic acid stain 4,6-diamino-2-phenylindole (DAPI) was used. Cells were

manually counted using a Nikon Eclipse E400 fluorescent microscope (Nikon Instruments Europe, Kingston, UK). A minimum of 15 random fields were counted in each slide. Short-chain fatty acids (SCFA) were analysed as indicated by Sanz, Gibson, & Rastall. (2005) using an HPLC system (Hewlett-Packard HP1050 series, Agilent Technologies, Edinburgh, UK) equipped with a UV detector and an automatic injector. The column was an ion-exclusion Aminex HPX-87H (7.8 x 300 mm, Bio-Rad, Hertfordshire, UK) maintained at 50 °C. The eluent was 0.005 mmol L^{-1} sulfuric acid in HPLC grade water, and the flow was 0.6 mL min^{-1}. Detection was performed at 210 nm, and data were acquired using Chem Station for LC3D software (Agilent Technologies).

2.5 Statistical analysis.

Each bacterial group and SCFA was compared by using one-way ANOVA test followed by a Scheffe test as a post hoc comparison of means ($P<0.05$) using Statistica for Windows version 6 (2002) by Statsoft Inc. (Tulsa, OK, USA).

3. Results and Discussion

3.1. Characterization of the β-LG:GOS conjugates.

Bovine β-LG was efficiently glycated with GOS after the optimization of incubation conditions in order to obtain the maximum formation of the corresponding Amadori compounds, as previously shown (Sanz, Cote, Gibson, & Rastall., 2006). Before simulated

GI digestion, free GOS were successfully removed from the β-LG:GOS conjugates by diafiltration. GC-MS analyses of initial GOS concentration and unconjugated GOS after storage revealed that the ratio β-LG:GOS was approximately 2:1 (w:w). This information was necessary to utilize an equivalent proportion of free GOS in the positive controls used for fermentation in the batch culture systems with respect to the GOS linked to the peptidic chain by the glycation process.

β-LG:GOS samples were submitted to GI digestion and were analysed by LC-MS^n, identifying, as published before (Moreno et al., 2008), nineteen peptides glycated with GOS of different polymerization degree (up to 7 hexose units). These samples were used for *in vitro* incubation with faecal inoculums.

3.2. Changes in bacterial populations during in vitro batch culture fermentation.

Table 1 shows changes in bacterial populations during the incubation of β-LG:GOS, GOS and digested β-LG with the faecal samples. The total bacterial population increased with all treatments, but this effect was only significant after 24 h. A significant increase in bifidobacteria population was observed after both 10 and 24 h for free GOS and for the digested β-LG:GOS conjugates. However, no significant differences in the growth of these bacteria were observed between these samples. On the contrary, bifidobacteria did not show a significant growth during incubation with the unconjugated digested β-LG. Regarding *Lactobacillus-Enterococcus* group population, an increase during incubation with GOS, β-LG:GOS and β-LG at 24h of fermentation was observed, although no significant changes at 10h were detected. Counts of *Bacteroides* and *Clostridium coccoides -Eubacterium rectal*

group were increased with all treatments, these changes being more notable for GOS and β-LG:GOS at 24 h and for β-LG:GOS at 10 and 24 h, respectively. On the other hand, *Clostridium histolyticum group clusters I, II,* only showed significant differences at 24h of fermentation with GOS and β-LG:GOS as compared to the control, whereas *Atopobium* population did not showed significant differences during the fermentation in all treatments.

The bifidogenic *in vitro* response of the colonic microflora to GOS has been well documented (Palframan, et al., 2003; Tzortzis, et al., 2004; Vulevic, et al., 2004), but the selectivity of these carbohydrates conjugated with food proteins has not been reported. Our results indicate that the bifidogenic properties of GOS were retained after glycation. Furthermore, bacteroides, clostridia and atopobium populations had a similar behaviour after fermentation of digested β-LG:GOS conjugates as compared to that of the well-known prebiotic GOS. Taken together, these results reinforce the fact that prebiotic properties of GOS, in terms of bacteria selectivity, are not modified following its glycation under controlled conditions. Similar results were found by Huebner et al. (2008), although these authors studied fructan-type oligosaccharides conjugated with glycine after heating at high; consequently, compounds different from those of Amadori should be formed in notable amounts. Moreover, the prebiotics tested by Huebner et al. (2008) contained remaining mono- and disaccharides, such as sucrose, glucose and fructose and the authors concluded that it was likely that these carbohydrates were responsible for the formation of MR products.

3.3. SCFA and lactic acid production.

Table 2 shows the values obtained for lactic acid and SCFA production during the incubation with the different tested subtracts. Only acetic, lactic and propionic acids were detected in the samples. A significant increase of lactic and acetic acids was observed in the presence of GOS at 10 and 24 h, whereas only lactic acid significantly increased for β-LG and β-LG:GOS. Acetic and lactic acids are fermentation end-products of the bifidobacteria pathways (Sanz et al., 2005; Sanz et al., 2006), however, it is difficult to relate the production of these acids with one bacterial genus when mixed cultures are studied. On the other hand, no significant differences were observed for propionic acid.

4. Conclusions

The glycation of β-LG with GOS under controlled conditions did not affect the *in vitro* fermentation selectivity of these carbohydrates. Previous research has demonstrated the undigestibility of the Amadori compound, which may conceivably reach the distal region of the colon (Erbersdobler, & Faist, 2001; Faist, & Erbersdobler, 2001; Finot, 2005). In consequence, although *in vivo* studies should be conducted, glycation involving prebiotic carbohydrates and peptides might be an alternative to obtain a new generation of prebiotics with enhanced colonic persistence. Lastly, it would be worth exploring the potential combination of the prebiotic properties with other bioactivities provided by the peptidic sequence.

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Vulevic, J., Rastall, R. A., & Gibson, G. R. (2004). Developing a quantitative approach for determining the in vitro prebiotic potential of dietary oligosaccharides. *FEMS Microbiology Letters, 236*, 153-159.

297 **Table 1**. Bacterial populations (log 10 cells mL^{-1} batch culture) in pH controlled cultures at 0, 10 and 24 hours of fermentation using

298 GOS, β-LG:GOS and β-LG.

Treatment	Time (h)	Total cells	Bifidobacteria	Bacteroides	*Lactobacillus-Enterococcus* group	*Clostridium histolyticum* group clusters I, II,	Atopobium group	*Clostridium coccoides - Eubacterium rectal* group
Control	0	9.02 (0.02)[§ b*]	7.83 (0.01)[a]	8.00 (0.07)[d]	7.01 (0.11)[abc]	6.75 (0.05)[a]	7.97 (0.48)[a]	7.24 (0.06)[c]
No treatment	10	9.50 (0.02)[ab]	8.06 (0.01)[a]	8.09 (0.02)[cd]	6.91 (0.04)[a]	7.32 (0.01)[ab]	7.85 (0.0)1[a]	8.02 (0.20)[a]
	24	9.78 (0.05)[ac]	7.92 (0.07)[a]	8.41 (0.07)[ab]	6.95 (0.16)[ab]	6.97 (0.03)[a]	7.32 (0.13)[a]	7.77 (0.22)[ab]
GOS	10	9.42 (0.02)[ab]	9.29 (0.07)[b]	8.41 (0.08)[ab]	7.12 (0.20)[abc]	7.30 (0.22)[ab]	7.45 (0.01)[a]	7.51 (0.08)[bc]
	24	9.83 (0.01)[ac]	9.07 (0.05)[b]	8.76 (0.05)[e]	7.50 (0.04)[d]	7.69 (0.06)[b]	7.29 (0.14)[a]	7.92 (0.04)[ab]
β-LG:GOS**	10	9.45 (0.10)[ab]	9.18 (0.04)[b]	8.42 (0.08)[ab]	7.12 (0.11)[abc]	7.12 (0.22)[ab]	7.79 (0.08)[a]	8.17 (0.11)[a]
	24	10.03 (0.11)[c]	9.19 (0.04)[b]	8.59 (0.07)[be]	7.38 (0.04)[bcd]	7.68 (0.06)[b]	7.62 (0.08)[a]	8.11 (0.06)[a]
β-LG**	10	9.43 (0.04)[ab]	8.23 (0.10)[a]	8.32 (0.08)[acd]	6.87 (0.10)[a]	7.06 (0.14)[ab]	7.54 (0.09)[a]	7.51 (0.14)[bc]
	24	9.91 (0.11)[ac]	8.32 (0.11)[a]	8.33 (0.03)[ac]	7.42 (0.10)[cd]	7.29 (0.33)[ab]	7.43 (0.26)[a]	7.94 (0.11)[ab]

299 *Different letters indicate significant differences ($P \leq 0.05$) for each bacterial group.

300 ** Samples had been submitted to *in vitro* gastrointestinal digestion

301 § Standard deviation (n=3).

Table 2. SCFA and lactic acid concentration (mM) in pH controlled cultures at 0, 10 and 24 hours of fermentation using GOS, β-LG:GOS and β-LG.

	Time	Lactic *acid*	Acetic *acid*	Propionic *acid*
Control	0	0.00 (0.00)[§a*]	1.01 (0.24)[b]	0.76 (0.20) [ab]
No treatment	10	0.00 (0.00)[a]	4.87 (0.61)[a]	0.78 (0.68) [ab]
	24	0.00 (0.00)[a]	5.23 (0.00)[b]	0.00 (0.00)[a]
GOS	10	3.71 (0.29)[e]	27.94 (9.53)[c]	1.28 (0.53) [ab]
	24	3.44 (0.00)[f]	30.99 (7.80)[c]	3.48 (1.67)[b]
β-LG	10	2.16 (0.32)[b]	5.80 (0.69)[a]	1.19 (0.25)[ab]
	24	1.53 (0.29)[c]	6.39 (1.78)[ab]	2.25 (1.54)[ab]
β-LG:GOS	10	2.39 (0.50)[b]	9.07 (3.40)[a]	1.06 (0.11)[ab]
	24	1.87 (0.04)[d]	6.52 (1.92)[ab]	2.02 (0.86)[ab]

[*]Different letters indicate significant differences ($P \leq 0.05$) for each acid.

[§] Standard deviation.