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In Vitro Fermentation by Human Gut Bacteria of Proteolytically Digested Caseinomacropeptide Nonenzymatically Glycosylated with Prebiotic Carbohydrates

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ABSTRACT: The in vitro fermentation selectivity of hydrolyzed caseinomacropeptide (CMP) glycosylated, via Maillard reaction (MR), with lactulose, galacto-oligosaccharides from lactose (GOSLa), and galacto-oligosaccharides from lactulose (GOSLu) was evaluated, using pH-controlled small-scale batch cultures at 37 °C under anaerobic conditions with human feces. After 10 and 24 h of fermentation, neoglyconjugates exerted a bifidogenic activity, similar to those of the corresponding prebiotic carbohydrates. No significant differences were found in *Bacteroides*, *Lactobacillus*−*Enterococcus*, *Clostridium histolyticum* subgroup, *Atopobium* and *Clostridium coccoides*−*Eubacterium rectale* populations. Concentrations of lactic acid and short-chain fatty acids (SCFA) produced during the fermentation of prebiotic carbohydrates were similar to those produced for their respective neoglycoconjugates at both fermentation times. These findings, joined with the functional properties attributed to CMP, could open up new applications of MR products involving prebiotics as novel multiple-functional ingredients with potential beneficial effects on human health.

KEYWORDS: Maillard reaction, Amadori compound, caseinomacropeptide, prebiotics, lactulose, galacto-oligosaccharides, bifidogenic

INTRODUCTION

Caseinomacropeptide (CMP) is composed of the 64 C-terminal amino acids of κ-casein released by chymosin or pepsin cleavage during the manufacture of cheese or digestion in the stomach. CMP comprises a heterogeneous group of polypeptides that differ, mainly, in the degree of glycosylation and phosphorylation.[1] Many biological properties have been attributed to CMP either in its intact form or after enzymatic hydrolysis.[2,3] Several works have reported the capacity of bovine CMP to interact with pathogens, viruses, bacteria, and toxins, depending on the content and structure of the glycans present in the amino acidic sequence.[4,5] Initially, the in vitro bifidogenic effect of intact CMP was reported by using pure cultures.[6−8] However, this effect was not later supported by Brück et al.[9] using breast milk supplemented with CMP and fermented with infant fecal slurries, although a significant decrease in *Clostridium histolyticum*, *Bacteroides*, and *Escherichia coli* was reported. Similarly, the prebiotic effect of intact CMP-supplemented infant formulas could not be demonstrated after ingestion by healthy term infants initially breast-fed.[10]

Currently, the Maillard reaction is used to improve the functionality of food proteins without requiring the addition of chemical reagents.[11] Different technological applications based on emulsifying, textural, and solubility properties of proteins have been considered, although the effect on biological properties, such as the selective fermentation of gut microflora, is also gaining great importance. However, there is currently very little and controversial information on this topic. Ames et al.[12] reported the nonspecific increase of anaerobic bacteria after fermentation of melanoidins produced from an aqueous glucose−lysine model system, whereas Borrelli and Fogliano[13] observed that bread crust melanoidins stimulated the growth of bifidobacteria. Later, an increase of some detrimental species of bacteria (sulfate-reducing bacteria and clostridia) and a decrease of bifidobacteria and lactobacilli was found after using bovine serum albumin (BSA) glycated with glucose as substrate of fermentation with human fecal bacteria compared to native BSA.[14] Maillard reaction (MR) products derived from roasted cocoa bean reduced the growth of pathogenic bacteria (*E. coli* spp. and *Enterobacter cloacae*) and bifidobacteria.[15]

Nevertheless, to the best of our knowledge, there is no information on the fermentation selectivity of proteins or peptides conjugated with prebiotic carbohydrates. Huebner et al.[16] studied the effect of MR conditions on the prebiotic activity of different commercial fructooligosaccharides conjugated with glycine using pure culture assays. However, these prebiotics were not previously fractionated and the formation of the MR products could be attributed to the presence of minor reducing sugars such as glucose and fructose.

It should be taken into consideration that in vitro and in vivo studies have shown that nondigested Amadori compounds, the first stable products of the MR, could reach the colon, where they can be fermented by microorganisms.[17−19] As a consequence, the conjugation between food proteins or peptides and prebiotic

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carbohydrates could potentially allow carbohydrates to reach the distal parts of the colon, where many chronic gut disorders originate.[20] Furthermore, increasing attention is being focused on the production of ingredients with multiple functional properties. Therefore, the conjugation of prebiotic carbohydrates with proteins or peptides possessing widely recognized functional properties can be of great interest. In this work, we have investigated the effect of a fraction rich in Amadori compounds derived from the glycation under controlled conditions of previously proteolytically digested CMP with three different carbohydrates (lactulose, galacto-oligosaccharides (GOS) from lactose, and novel GOS from lactulose) on the human intestinal microbiota as represented by feces, using small-scale in vitro batch culture.

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich (Dorset, U.K.), and the bacteriological growth medium supplements were obtained from Oxoid (Basingstoke, U.K.). Bovine caseinomacropeptide (CMP) was kindly provided by Davisco Foods International, Inc. (Le Sueur, MN). Galacto-oligosaccharides from lactose (GOSLa) were obtained from Vivinal-GOS, kindly provided by Friesland Foods Domo (Zwolle, The Netherlands). This product had 73 wt % dry matter, the composition of which was 60 wt % GOS, 20 wt % lactose, 19 wt % glucose. and 1 wt % galactose, as stated by the supplier. Duphalac (Solvay Pharma, Brussels, Belgium) was used to obtain the galacto-oligosaccharides from lactulose (GOSLu). Duphalac has 67% lactulose (w/v), <11% galactose (w/v), <6% lactose (w/v), <4.7% epilactose (w/v), and <2% tagatose (w/v), as stated by the supplier.

Preparation of Galacto-oligosaccharides. To remove digestible and nonprebiotic mono- and disaccharides and obtain oligosaccharides (GOSLa), the commercial product Vivinal-GOS was fractionated using size exclusion chromatography, following the method reported by Hernandez et al.[21] with some modifications. In brief, 80 mL of Vivinal-GOS (25% w/v) was injected in a preparative Bio-Gel P2 (Bio-Rad, Hercules, CA) column (90 × 5 cm) using water as mobile phase, at 1.5 mL min^{-1}. Sixty fractions of 10 mL were collected, after the elution of void volume. The degree of polymerization (DP) of fractions was determined by electrospray ionization mass spectrometry (ESI-MS) at positive mode. Fractions with DP ≥ 3 were pooled and freeze-dried.

GOS from lactulose (GOSLu) were obtained following the method previously described.[22] A solution (450 g/L) of Duphalac was dissolved in 50 mM sodium phosphate buffer and 1 mM $MgCl_2$, pH 4.5, after addition of 8 U/mL of β-galactosidase from *Aspergillus oryzae* (Sigma-Aldrich) and incubation at 60 °C for 8 h under continuous agitation at 300 rpm. Following incubation, the mixtures were immediately immersed in boiling water for 5 min to inactivate the enzyme. After enzyme inactivation, the mixture was purified following the method proposed by Morales et al.[23] with some modifications, by dilution (1/50) with water and treatment with 48 g of activated charcoal per 40 g of initial carbohydrates to remove monosaccharides. Oligosaccharides (GOSLu) were recovered using ethanol/water 50:50 (v/v), the ethanol was evaporated using a rotavapor, and the remaining solution was freeze-dried. The DP of the sample was determined by ESI-MS at positive mode, containing carbohydrates with DP ≥ 2.

Preparation of Glycoconjugates. A previous step of hydrolysis of CMP was necessary to increase the number of free primary amino groups available for glycation and, consequently, to yield potentially and highly functional food-grade peptide−carbohydrate conjugates. Thus, CMP was hydrolyzed following the method proposed by Hernandez-Hernandez et al.,[24] using a combination of trypsin/chymotrypsin (overnight at 37 °C and pH 7) at 1:0.05:0.025, CMP/trypsin/chymotrypsin ratios (w/w/w). Activities of porcine pancreas trypsin (EC 3.4.21.4, type IX-S) and bovine pancreas α-chymotrypsin (EC 3.4.21.1, type I-S) were 13000−20000 and ≥40 U mg^{-1} of protein, respectively. Enzymes were inactivated by heating at 95 °C for 5 min. The hydrolyzed CMP was freeze-dried.

Aliquots of a solution consisting of 60 mg mL^{-1} of hydrolyzed CMP and 60 mg mL^{-1} of the individual prebiotic carbohydrates (i) lactulose, (ii) GOSLa, and (iii) GOSLu dissolved in 0.1 M sodium phosphate buffer, pH 7.0, were lyophilized. These were kept under vacuum in a desiccator at 40 °C and a water activity of 0.44, achieved with a saturated K_2CO_3 solution (Merck, Darmstadt, Germany), for 9, 16, and 18 days. In addition, control experiments were performed with hydrolyzed CMP stored at 40 °C without carbohydrates during the same periods (heated CMP). Incubations were performed in duplicate, and all analytical determinations were performed at least in duplicate. 2-Furoylmethylamino acids (2-FM-AA) were determined to study the formation of Amadori compounds during Maillard reaction according to Moreno et al.[25] A previous hydrolysis of the samples using 8 N HCl at 110 °C for 23 h under inert conditions (helium) was carried out. Samples were filtered through Whatman no. 40 filter paper and purified through a Sep-Pak C18 cartridge previously activated with 5 mL of methanol and 10 mL of deionized water (Waters, Milford, MA); 2-FM-AA was eluted with 3 mL of 3 N HCl. Analyses were carried out by an ion-pair RP-HPLC method using a C8 (Alltech furosine-dedicated; Alltech, Nicolasville, KY) column (250 × 4.6 mm i.d.) and a variable-wavelength detector at 280 nm (LDC Analytical, SM 4000, Salem, NH). Operating conditions were as indicated by Resmini et al.[26] Quantitative analyses were performed by using known concentrations (from 0.52 to 5.2 mg L^{-1}) of a commercial pure standard of furosine (2-furoylmethyllysine; Neosystem Laboratories, Strasbourg, France).

After storage, the unconjugated carbohydrates were quantified by gas chromatography (GC-FID) using a previous two-step derivatization procedure: oximation and trimethylsilylation.[27] The samples were mixed with phenyl-β-glucoside as internal standard and evaporated under vacuum. The oximes were formed using 350 μL of hydroxylamine hydrochloride in pyridine (2.5% w/v) and then silylated using 350 μL of hexamethyldisilazane and 35 μL of trifluoroacetic acid. GC-FID analyses were carried out using an HP-7890A chromatograph (Hewlett-Packard, Palo Alto, CA) and nitrogen as a carrier gas. Carbohydrates were separated using an HT5 column (25 m × 0.22 μm × 0.1 μm) coated with 5% phenyl polysiloxane-carborane (SGE Europe, Milton Keynes, U.K.). Oven temperature was programmed as indicated by Hernandez et al.[21]

The unconjugated carbohydrate fraction was removed by ultrafiltration at 4 °C using a diafiltration unit model 8400 (Millipore) with a molecular cutoff of 1 kDa and, then, the retentate was analyzed by ESI-MS to confirm the absence of unconjugated carbohydrates.

The quantity of conjugated prebiotic was determined considering the differences between the amounts used at the initial step of the glycation process and the unconjugated carbohydrate fraction determined by GC-FID. Thus, equivalent amounts of the corresponding free prebiotics were used for the in vitro fermentation assays to accurately compare with the neoglycoconjugates.

Fecal Samples and in Vitro Fermentations. Fecal samples were obtained from three healthy donors (one male and two females; 25−30 years old) without any known metabolic or gastrointestinal disorders. Samples were collected and kept in an anaerobic cabin for a maximum of 15 min. The samples were diluted (1/10 w/w) with phosphate buffer (0.1 M, pH 7.4) and homogenized in a stomacher for 2 min at normal speed.

Gently stirred pH-controlled small-scale fermenters (5 mL working volume) were filled with basal nutrient medium (peptone water 2 g L^{-1}, yeast extract 2 g L^{-1}, NaCl 0.1 g L^{-1}, K_2HPO_4 0.04 g L^{-1}, KH_2PO_4 0.04 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.01 g L^{-1}, $CaCl_2 \cdot 6H_2O$ 0.01 g L^{-1},

$NaHCO_3$ 2 g L^{-1}, Tween 80 2 mL, hemin 0.02 g L^{-1}, vitamin K_1 10 μL, cysteine-HCl 0.5 g L^{-1}, bile salts 0.5 g L^{-1}, pH 7.0) and gassed overnight with nitrogen. After the addition of fecal solution (final concentration = 1% v/v), samples (neoglycoconjugates or free prebiotics), previously sterilized by filtration through 0.22 um filters, were added to a final concentration of 1% w/v. The amount of CMP/prebiotic neoglycoconjugates was determined considering the initial amount of hydrolyzed CMP and the conjugated prebiotic fraction calculated as described above. Additionally, a control vessel without substrate was included (negative control).

The temperature was kept at 37 °C at a pH between 6.7 and 6.9 and gassed with nitrogen during the entire fermentation process. Fermentations were run over a period of 24 h, and 1 mL samples were taken at 0, 10, and 24 h for fluorescent in situ hybridization (FISH) and HPLC analyses as described below.

Bacterial Enumeration by Fluorescent in Situ Hybridization (FISH). The FISH technique was used to quantify some bacterial groups and, in consequence, to monitor changes in bacterial populations. The samples obtained from the fermentations were fixed (1/4) in 4% (w/v) paraformaldehyde for 6 h at 4 °C. Samples were then centrifuged at 1500*g* for 10 min and washed twice with phosphate-buffered saline (PBS, 0.1 M, pH 7.0). The obtained pellet was resuspended in cold PBS solution and ethanol (99%) (1:1 v/v) and stored for at least 1 h at −20 °C.

The samples were diluted with PBS to obtain 10−100 cells per field of view to count after hybridization. The hybridization was carried out following the method proposed by Martin-Pelaez et al.[28] using 16S rRNA-targeted oligonucleotide probes labeled with Cy3. Probes, commercially available (Sigma-Aldrich, Dorset, U.K.), were as follows: Bif164, specific for *Bifidobacterium*;[29] Bac303, specific for *Bacteroides*;[30] Chis150, for the *Clostridium histolyticum* group clusters I and II;[31] Erec482 for the *Clostridium coccoides*−*Eubacterium rectale* group;[31] Lab158 for the *Lactobacillus*−*Enterococcus* group;[32] and Ato291 for the *Atopobium* cluster.[32] For total counts the nucleic acid stain 4,6-diamino-2-phenylindole (DAPI) was used. Samples were examined under a fluorescent microscope (Nikon Eclipse, E400), and DAPI-stained cells were examined under ultraviolet light. A minimum 15 random fields of view were counted for each sample.

Short-Chain Fatty Acids (SCFA) and Lactic Acid Analyses. Samples from the fermenters were centrifuged at 13000*g* for 10 min to remove all particles, and the produced lactic, acetic, propionic, and butyric acids were quantified using a Bio-Rad HPX-87H HPLC column (Watford, U.K.) at 50 °C, with a 0.005 mM H_2SO_4 as mobile phase, in isocratic mode, at a flow rate of 0.6 mL/min.[33]

Statistical Analyses. Statistical analyses was performed using Statistica for Windows version 6 (2002) by Statsoft Inc. (Tulsa, OK). Differences between bacterial counts and SCFA concentration were tested using one-way ANOVA test, followed by Scheffe test as a post hoc comparison of means ($P < 0.05$).

RESULTS AND DISCUSSION

Determination of the Extent of Glycation between Prebiotic Carbohydrates and CMP. Maillard reaction between lactulose, GOSLa, and GOSLu and hydrolyzed CMP was controlled by studying the formation of 2-FM-AA, which is formed upon acid hydrolysis of the peptide-bound Amadori products. The formation of 2-FM-AA was measured as milligrams of furosine (2-furoylmethyl-lysine) per 100 mg of neoglycoconjugate. Maximum yields were obtained after 16 days of incubation for CMP:GOSLu (1.57 mg of furosine per 100 mg of neoglycoconjugate) and for CMP:GOSLa (1.90 mg of furosine per 100 mg of neoglycoconjugate) (Figure 1). It has been described that, in general terms, aldoses react more rapidly than ketoses to form the corresponding Schiff base prior to its rearrangement to the more stable Amadori or Heyns compound, because the aldehyde carbonyl groups are relatively more electrophilic than ketone carbonyl groups.[34] As a consequence, the higher yield obtained for CMP:GOSLa can be due to the presence of an aldose (mostly glucose) at the reducing end of GOSLa,[35] in comparison with GOSLu that presents mainly a ketose (fructose) at the reducing end.[36] Finally, after 16 days of incubation the levels of furosine decreased in CMP:GOSLa glycoconjugates, which was indicative of the predominant degradation of Amadori compounds to dicarbonyl intermediates that may serve as precursors of the advanced stages of the Maillard reaction, whereas it remained constant in CMP:Lu and CMP:GOSLu (Figure 1).

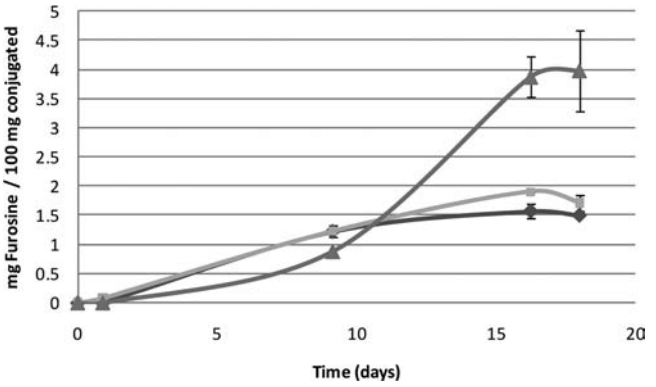

Figure 1. Furosine content at different storage times during the glycation of hydrolyzed CMP with lactulose (▲) GOSLu (◆), and GOSLa (■).

Furthermore, the yield after 16 days of incubation for CMP:lactulose was much higher (3.87 mg of furosine per 100 mg of glycoconjugate) than those obtained for the neoglycoconjugates based on GOSLa or GOSLu (Figure 1). This behavior is explained by the well-established order of reactivity according to which monosaccharides are more reactive than disaccharides and these more reactive than oligosaccharides due to the smaller the carbonic chain of the sugar is, the more acyclic forms exist and the more reactive is the sugar with the amino groups of proteins.[37,38]

Likewise, GC-MS analyses of initial GOS concentration and unconjugated carbohydrates after storage revealed that the relation CMP:GOSLu, CMP:GOSLa, and CMP:lactulose was approximately 2:1 (w/w).

Changes in Bacterial Populations during in Vitro Batch Culture Fermentation. Table 1 shows changes in bacterial population after fermentation of the neoglyconconjugates, free carbohydrates, hydrolyzed CMP, and corresponding controls with fecal samples for 10 and 24 h. The bacterial composition of the inoculum is likely to be affected by several factors such as genetics, age, health status, nutrition, and diet of the volunteers.[39] Quantitative results obtained for the three donors had a low standard deviation, which is indicative of the data consistency presented in this work. In general, no significant differences were found in total cells, Bac303, Lab158, Chis150, Ato291, and Erec482 for all samples under study. Similar behavior has been reported for lactulose and GOS from lactose by Rycroft et al.[40] after 24 h of fermentation.

Bif164 values detected for hydrolyzed CMP after both 10 and 24 h of incubation did not show significant differences with the control (Table 1). The bifidogenic activity of intact CMP had been previously reported by using pure cultures,[6−8,41] although

Table 1. Bacterial Populations (Log 10 Cells per Milliliter of Batch Culture) in pH-Controlled Cultures at 0, 10, and 24 h of Fermentation Using Glycated Hydrolyzed CMP with Lactulose (CMP:Lactulose), Galacto-oligosaccharides from Lactulose (CMP:GOSLu), and Galacto-oligosaccharides from Lactose (CMP:GOSLa) and Their Corresponding Free Carbohydrates and Control (No Substrate)[a]

sample	time (h)	total cells	Bif164	Bac303	Lab158	Chis150	Ato291	Erec482
control	0	9.02 (0.02)[b] a	7.83 (0.01) a	8.00 (0.07) a	7.01 (0.11) a	6.75 (0.05) a	7.97 (0.48) a	7.24 (0.06) a
	10	9.50 (0.02) ab	8.06 (0.01) ab	8.09 (0.02) a	6.91 (0.04) a	7.32 (0.01) abc	7.85 (0.0)1 a	8.02 (0.20) a
	24	9.78 (0.05) b	7.92 (0.07) a	8.41 (0.07) a	6.95 (0.16) a	6.97 (0.03) abc	7.32 (0.13) a	7.77 (0.22) a
lactulose	10	9.33 (0.12) ab	9.21 (0.08) d	8.48 (0.10) a	7.06 (0.14) a	7.23 (0.16) abc	7.65 (0.06) a	7.55 (0.06) a
	24	9.73 (0.08) b	8.85 (0.12) cd	8.52 (0.06) a	7.22 (0.03) a	7.73 (0.06) bc	7.53 (0.03) a	7.95 (0.03) a
GOSLu	10	9.33 (0.06) ab	9.18 (0.05) d	8.48 (0.07) a	7.09 (0.09) a	7.20 (0.07) abc	7.67 (0.06) a	7.54 (0.13) a
	24	9.73 (0.09) b	8.95 (0.08) cd	8.39 (0.07) a	7.38 (0.29) a	7.71 (0.04) abc	7.52 (0.04) a	7.97 (0.12) a
GOSLa	10	9.42 (0.02) ab	9.29 (0.07) d	8.41 (0.08) a	7.12 (0.20) a	7.30 (0.22) abc	7.45 (0.01) a	7.51 (0.08) a
	24	9.83 (0.01) b	9.07 (0.05) cd	8.76 (0.05) a	7.50 (0.04) a	7.69 (0.06) abc	7.29 (0.14) a	7.92 (0.04) a
hydrolyzed CMP	10	9.42 (0.04) ab	8.41 (0.19) bc	8.29 (0.08) a	6.88 (0.21) a	7.37 (0.13) abc	7.67 (0.03) a	7.99 (0.14) a
	24	9.78 (0.09) b	8.13 (0.11) abc	8.51 (0.08) a	6.86 (0.10) a	7.26 (0.16) abc	7.29 (0.15) a	7.69 (0.16) a
CMP:lactulose	10	9.07 (0.53) ab	8.71 (0.74) cd	8.14 (0.73) a	6.89 (0.41) a	6.82 (0.61) ab	7.51 (0.64) a	7.17 (0.51) a
	24	9.44 (0.50) ab	8.52 (0.69) cd	7.99 (0.60) a	7.11 (0.48) a	7.38 (0.66) abc	7.33 (0.62) a	7.59 (0.54) a
CMP:GOSLu	10	9.28 (0.05) ab	9.12 (0.03) cd	8.54 (0.01) a	7.20 (0.13) a	7.11 (0.03) abc	7.71 (0.15) a	7.59 (0.01) a
	24	9.73 (0.06) b	8.81 (0.03) cd	8.83 (0.03) a	7.37 (0.20) a	7.64 (0.09) abc	7.51 (0.11) a	8.02 (0.02) a
CMP: GOSLa	10	9.38 (0.05) ab	9.21 (0.01) d	8.56 (0.13) a	7.09 (0.08) a	7.23 (0.17) abc	7.56 (0.20) a	7.56 (0.15) a
	24	9.76 (0.08) b	8.85 (0.11) cd	8.72 (0.04) a	7.22 (0.01) a	7.97 (0.05) c	7.48 (0.11) a	7.95 (0.02) a

[a] Different letters indicate significant differences ($P \leq 0.05$) for each bacterial group. [b] Standard deviation ($n = 3$).

this activity could not be further confirmed following studies with mixed cultures from infant feces.[9,10] Our results support the nonbifidogenic effect described for the intact CMP, stressing the importance of carrying out mixed culture studies for the evaluation of prebiotic properties. In addition, to the best of our knowledge, this is the first evidence showing the effect on human gut bacteria following in vitro fermentation of previously proteolytically digested CMP.

On the contrary, a significant increase in the Bif164 population at both incubation times was detected for the free carbohydrates (lactulose, GOSLu, and GOSLa) and their corresponding neoglycoconjugates (CMP:lactulose, CMP:GOSLa, and CMP:GOSLu) (Table 1). This result confirmed the bifidogenic effect of these carbohydrates as previously reported.[33,40,42,43] Furthermore, it is worth noting that the three neoglyconjugates showed a bifidogenic effect similar to those obtained with the corresponding free prebiotics, indicating that the glycation of CMP with prebiotics did not affect their bifidogenic activity. A possible explanation for this behavior may be that for the formation of the corresponding Amadori or Heyns compounds, the prebiotic moieties do not change drastically because only the carbonyl group reacts with the free primary amino group from peptides, through a condensation of both groups, forming a glycosylamine that undergoes Amadori or Heyns rearrangement. Accordingly, Huebner et al.[16] found that the prebiotic score of fructooligosaccharides did not decrease after their conjugation with glycine.

Changes in Lactic Acid and SCFA Profile. Table 2 shows the values obtained for lactic acid and SCFA produced during the incubation with the different carbohydrates and neoglycoconjugates tested. Acetic acid was the most abundant SCFA in all studied samples and, with the exception of CMP:lactulose conjugate, a notable increase was detected after 10 h of fermentation. In addition, the formation of this acid was more highly promoted by the prebiotic carbohydrates than by the neoglycoconjugates. Lactic acid was detected only at 10 h of fermentation of carbohydrates and neoglycoconjugates. A possible cause for the decrease in this acid during fermentation process could be the fact that it can be converted into other SCFA by different bacteria genera.[44,45] The concentrations of lactic acid did not show significant differences for GOSLu with its respective neoglycoconjugate. On the contrary, in the treatment with GOSLa, the concentration of lactic acid was higher than that of CMP:GOSLa, whereas for lactulose the concentration of this acid was significantly lower than that of CMP:lactulose. Both lactic and acetic acids are considered to be end products of the bifidobacteria pathway, supporting the increase in bifidobacteria population described in Table 1. However, it cannot establish a linear relationship between production of SCFA and bacterial genus, considering that several bacteria are involved in the production of these acids.

An increase of propionic acid was found at 24 h of fermentation for all samples tested compared with 10 h of fermentation. No significant differences between prebiotic carbohydrates and the corresponding neoglyconjugates were found at both

Table 2. Lactic Acid and SCFA in pH-Controlled Cultures at 0, 10, and 24 h of Fermentation Using Glycated Hydrolyzed CMP with Lactulose (CMP:Lactulose), Galacto-oligosaccharides from Lactulose (CMP:GOSLu), and Galacto-oligosaccharides from Lactose (CMP:GOSLa) and Their Corresponding Free Carbohydrates and Control (No Substrate)[a]

		mM			
sample	time (h)	lactic acid	acetic acid	propionic acid	butyric acid
control	0	0.00 a	0.00 a	0.00 a	0.00 a
	10	0.00 a	0.00 a	0.00 a	0.00 a
	24	0.00 a	0.00 a	0.00 a	0.00 a
lactulose	10	6.82 (4.58)[b] b	27.07 (0.78) bc	3.11 (0.76) ab	0.00 a
	24	0.00 a	32.00 (9.22) cd	10.09 (0.37) de	0.00 a
GOSLu	10	4.06 (2.63) b	22.48 (1.61) bc	1.68 (0.75) a	0.00 a
	24	0.00 a	43.35 (3.69) d	9.78 (3.12) cde	0.00 a
GOSLa	10	13.69 (1.51) d	31.83 (4.57) cd	0.00 a	0.00 a
	24	0.00 a	62.85 (13.21) e	24.23 (4.21) g	0.00 a
hydrolyzed CMP	10	0.00 a	19.5 (5.26) bc	0.00 a	0.00 a
	24	0.00 a	23.47 (6.53) bc	12.75 (1.40) e	0.00 a
CMP:lactulose	10	10.48 (6.01) c	5.72 (0.86) a	3.70 (0.61) ab	0.00 a
	24	0.00 a	24.45 (14.98) bc	12.54 (1.38) e	0.47 (0.01) a
CMP:GOSLu	10	5.95 (0.19) b	18.89 (1.19) b	0.00 a	0.00 a
	24	0.00 a	34.88 (3.40) cd	6.51 (2.18) bc	0.00 a
CMP: GOSLa	10	4.33 (3.92) b	19.99 (1.56) bc	3.10 (0.35) ab	0.00 a
	24	0.00 a	29.00 (10.13) bc	19.72 (6.48) f	1.32 (0.37) a

[a] Different letters indicate significant differences ($P \leq 0.05$) for each acid. [b] Standard deviation ($n = 3$).

fermentation times, with the exception of GOSLa at 24 h. Finally, no significant formation of butyric acid was observed (Table 2), which can be explained by the fact that the butyric acid production is mostly related to changes enumerated by the Erec probe. As shown by Table 1, these bacteria did not significantly increase on these substrates.

In recent years, there has been a demand for production of a "second generation" of novel prebiotic ingredients that have a series of properties that include (i) displaying greater persistence in the large intestine, (ii) having great selectivity with regard to control of the intestinal microbiota (for example, to be metabolized by specific bifidobacteria), and (iii) having an additional biological activity, exerting beneficial effects on specific physiological functions and/or reducing the risk of disease, for example, through their effect on displacement of pathogens and regulation of the function of the immune system.[46] In this context, CMP: prebiotic neoglycoconjugates might represent potential candidates for second-generation prebiotics as this study has revealed that glycosylation via Maillard reaction of all assayed prebiotic carbohydrates with hydrolyzed CMP under controlled conditions does not reduce the bifidogenic properties of carbohydrates, and in the case of the neoglyconjugates with GOSLu and lactulose, the lactic acid and SCFA production was also comparable to that of free carbohydrates.

Consequently, these findings might represent a new alternative to produce novel multifunctional food ingredient by keeping the prebiotic properties of the carbohydrate portion and adding the biological properties of the peptide fraction. Nevertheless, further gut persistence and in vivo studies should be conducted to explore the potential health benefits of these neoglycoconjugates on animals and human volunteers, as well as to evaluate the impact of glycation on the protein/peptide structure and, subsequently, on its biological properties.

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