**Maillard-type glycoconjugates from dairy proteins inhibit adhesion
of *Escherichia coli* to mucin**

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Abstract

In this study, glycoconjugates of β -lactoglobulin (β -lg) and sodium caseinate (SC) were obtained via Maillard reaction with galactose and lactose, and their ability to inhibit adhesion of different *Escherichia coli* strains (CBL2, CBM1 and CBL8) to mucin was evaluated. The strains tested exhibited different interaction patterns with the glycoconjugates, suggesting the participation of different carbohydrate-recognition sites in adhesion. Galactosylation and lactosylation of both β -lg and SC significantly decreased the adhesion values of *E. coli* CBL2 to mucin. Whereas the adhesion of *E. coli* CBM1 was preferably interfered by galactosylated glycoconjugates obtained under the harshest incubation conditions, the adhesion capacity of *E. coli* CBL8 was not affected. Competitive adhesion assays with lectins, which recognize different epitopes, supported the idea that galactose-reactive adhesins are partly responsible for the recognition of these glycoconjugates. The analysis of the presence of gene coding for several virulence factors in the *E. coli* strains by PCR revealed the absence of K88 gene in the CBL2 strain assayed. These findings suggest that the formation of Maillard-type neoglycoproteins under controlled conditions may be a simple and cost-effective method for producing new food ingredients with the potential ability to block pathogen adhesins involved in mucosal colonization.

Keywords: dairy proteins, glycation, adhesion inhibition, *Escherichia coli*, functional foods.

1. Introduction

Many bacterial pathogens use proteins (lectin-like molecules) that bind to carbohydrates located at the surface of the host tissues to initiate infection (Zopf & Roth, 1996). Blocking these lectins with suitable carbohydrates that are structurally similar to the host receptors may be an effective strategy to prevent bacterial adhesion and infections (Karlsson, 1998; Ofek, Hasty & Sharon, 2003). Nevertheless, production of synthetic oligosaccharides may be extremely costly and to act effectively, large doses are normally required (Sharon, 2006). The low effectiveness of oligosaccharides to inhibit bacterial adhesion may be due to high absorption through the intestinal mucosa, and/or to low affinity for the bacterial lectins, which makes their use necessary at high concentrations (Ofek et al., 2003). Consequently, the attachment of many copies of the saccharide to suitable polymeric carriers, yielding multivalent adhesion sites, has been proposed as a novel approach to increase the affinity for the bacterial lectins (Sharon, 2006).

In this context, production of neoglycoproteins through the Maillard reaction, the so-called glycation, under controlled conditions could be an alternative for developing compounds with anti-adhesive properties. Many studies have demonstrated that glycation of proteins may be an effective, inexpensive and simple method to produce glycoconjugates with optimum functionality in real food systems (Oliver, Melton & Stanley, 2006a). Briefly, the Maillard reaction is initiated by a condensation between the carbonyl group of a reducing sugar and the free amino group of an amino acid, peptide or protein (ϵ -amino group of lysine and/or the α -amino group of terminal amino acid), which cyclizes to the *N*-substituted glycosylamine and is then rearranged to form the Amadori product. Further reactions, whose nature depends mainly on the amounts of oxygen, water, temperature and pH of the system, give rise to different compounds

that include reductones, furfurals, and a variety of other cyclic compounds. Brown polymers, so-called melanoidins, are the final products of the reaction (Olano & Martinez-Castro, 1996).

Metabolic studies have shown that Maillard reaction products are poorly digested and they reach the hind gut where they may be utilized by microorganisms (Erbersdobler & Faist, 2001; Faist & Erbersdobler, 2001; Finot, 2005). Therefore, it is plausible that some Maillard reaction glycoconjugates may be available and bind lectins-like molecules present on the surface of the gut pathogenic bacteria, thereby inhibiting bacterial adhesion to the intestinal mucosa. Only few studies have evaluated the interaction of glycated proteins with specific lectins. In this sense, undigested lactosylated serum albumin from different origin, such as human, bovine and porcine, showed recognition through the terminal galactose unit of the lactose by liver (Aring, Schlepperschaefer, Burkart & Kolb, 1989), plant lectins (Ledesma-Osuna, Ramos-Clamont & Vazquez-Moreno, 2008) and *Escherichia coli* K88 adhesins (Sarabia-Sainz, Ramos-Clamont, Candia-Plata & Vazquez-Moreno, 2009; Ledesma-Osuna, Ramos-Clamont & Vazquez-Moreno, 2009). Likewise, Hiramoto et al. (2004) showed that a variety of food protein-derived melanoidins strongly inhibited adhesion of *Helicobacter pylori* to urease-gastric mucins and its colonization in mice.

The objectives of this study were to characterize a set of glycoconjugates obtained by glycation of bovine sodium caseinate or β -lactoglobulin with galactose or lactose at different stages of the Maillard reaction, and to evaluate their ability to inhibit the adhesion of several intestinal *Escherichia coli* strains to mucin after simulated gastrointestinal digestion.

2. Materials and Methods

2.1. Preparation and purification of β -Lactoglobulin-galactose/lactose conjugates.

Carbohydrates, galactose (Gal) or lactose (Lac), and β -Lg (mixture of A and B variants) (Sigma-Aldrich, St. Louis, MO) in a weight ratio of 1:1 or 2:1, respectively, were dissolved in 0.1 M sodium phosphate buffer, pH 7 (Merck, Darmstadt, Germany), and lyophilized. The β -Lg-Gal powders were kept at 40 and 50 °C for 1 and 2 days, respectively, whilst the β -Lg-Lac powders were kept at 60 °C for 8 hours and 2 days, under a vacuum in a desiccator equilibrated at an a_w of 0.44, achieved with a saturated K_2CO_3 solution (Merck). In addition, control experiments were performed with β -Lg stored at 40, 50 and 60 °C without reducing sugars during the same periods (control heated β -Lg). Incubations were performed in duplicate, and all analytical determinations were performed at least in duplicate.

After incubation, the products were reconstituted in distilled water to a protein concentration of 1 mg/mL. To remove free carbohydrate, 2 mL portions were ultrafiltered through hydrophilic 3 kDa cutoff membranes (Centricon YM-3, Millipore Corp., Bedford, MA) by centrifugation at 1548g for 2 h. After removal of free Gal or Lac, samples were reconstituted in distilled water at a concentration of 2 mg/mL for further analysis (Corzo-Martinez, Moreno, Olano & Villamiel, 2008).

2.2. Preparation and purification of sodium caseinate-galactose/lactose conjugates.

Carbohydrates (Gal or Lac) and sodium caseinate (SC) (Rovita FN 5, Proveedora Hispano Holandesa, S.A., Barcelona, Spain) in a weight ratio of 0.2:1 were dissolved in

0.1M sodium phosphate buffer, pH 7.0 (Merck) and lyophilized (Corzo-Martinez, Moreno, Villamiel & Harte, 2010a). The SC-Gal powders were kept at 60 °C and 50 °C for 4 hours and 3 days, respectively, whilst the SC-Lac powders were kept at 60 °C for 8 hours and 1 day, under vacuum in a desiccator equilibrated at an a_w of 0.67 (Oliver, Melton & Stanley, 2006b), achieved with a saturated solution of CuCl₂ (Sigma–Aldrich,). In addition, control experiments were performed with SC stored at 50 and 60 °C without reducing sugars during the same periods (control heated SC). Incubations were performed in duplicate, and all analytical determinations were performed at least in duplicate. After incubation, free carbohydrates were removed as explained in subsection 2.1.

2.3. Evaluation of the progress of the Maillard reaction.

MALDI-TOF-MS analyses of β -lg:Gal/Lac conjugates were performed using a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed nitrogen laser ($\lambda=337$ nm, 3 ns pulse width, and 3 Hz frequency) and a delayed extraction ion source (Corzo-Martinez, Moreno, Olano & Villamiel, 2010b). Ions generated by the laser desorption were introduced into the flight tube (1.3 m flight path) with an acceleration voltage of 25 kV, 93% grid voltage, 0.05% ion guide wire voltage, and a delay time of 350 ns in the linear positive ion mode. Mass spectra were obtained over the m/z range 10–35 kDa. Myoglobin (horse heart) and carbonic anhydrase were used for external calibration and sinapinic acid (10 mg/mL in 0.3% trifluoroacetic acid/ acetonitrile, 70:30, v/v) as the matrix. Samples were mixed with the matrix at a ratio of approximately 1:15, and finally, 1 μ L of this solution was spotted onto a flat stainless-steel sample plate and dried in air.

LC/ESI-MS analyses of SC:Gal/Lac conjugates were performed on a Finnigan Surveyor pump with quaternary gradient system coupled on-line to a Finnigan Surveyor PDA photodiode array detector and to a Finnigan LCQ Deca ion trap mass spectrometer using an ESI interface as previously described by [Corzo-Martinez et al. \(2010a\)](#). Thus, prior to LC/ESI-MS separations, SC:Gal/Lac conjugates were dissolved in 1 mL of 0.1 M Tris/HCl pH 7.0 buffer (Sigma-Aldrich) containing 8 M urea (Riedel-de Haën, Seelze, Germany) and 20 mM dithiothreitol (DTT, Sigma-Aldrich) to give a final protein concentration of 10 mg/mL. After standing at 37°C for 1 hour, samples were diluted (1/3) with solvent A and filtrated with PVDF membranes (0.45 µm, Symta, Madrid, Spain). LC/ESI-MS experiments were carried out on a Finnigan Surveyor pump with quaternary gradient system coupled in-line to a Finnigan Surveyor PDA photodiode array detector and to a Finnigan LCQ Deca ion trap mass spectrometer using an ESI interface. Sample injections (7 µL) were carried out by a Finnigan Surveyor autosampler. All instruments were from Thermo Fisher Scientific (San José, CA, USA). RP-LC separations were carried out with a BioBasic-4 (100 mm x 2.1 mm, 5 µm) column (Thermo Fisher Scientific) at 25 °C using an adaptation of the method by Neveu, Mollé, Moreno, Martin & Léonil (2002). Separation was achieved at a flow rate of 0.2 mL/min and using 0.25% (v/v) of formic acid (analytical grade, Merck, Darmstadt, Germany) in double-distilled water (Milli-Q water, Millipore, Bedford, USA) as solvent A and 0.25% (v/v) of formic acid in acetonitrile (LC-MS Chromasolv® grade, Riedel-de Haën, Seelz, Germany) as solvent B. The elution program was applied as follows: at the start 20% B; after 2 min the percentage of B was linearly increased to 40% in 3 min; 40-50% B linear from 5 to 12 min; 50-80% B linear from 12 to 15min; 80% B isocratic from 15 to 20 min; ramped to original composition in 1 min; and then equilibrated for 15 min. The PDA detector operated in the

wavelength range 190–600 nm in order to gather UV-spectral data. The detection wavelength was set at 280 nm. The mass spectrometer spray voltage was set at 4.5 kV, heated capillary temperature at 220 °C, nitrogen (99.5% purity) was used as sheath (0.6 L min⁻¹) and auxiliary (6 L min⁻¹) gas, and helium (99.9990% purity) as the collision gas. Mass spectra were recorded in the negative ion mode. The LC-MS system, data acquisition and processing were managed by Xcalibur software (1.2 version, Thermo Fisher Scientific). Spectra of the protein peaks detected were deconvoluted using the BIOMASS deconvolution tool from BioWorks 3.1 software (Thermo Fisher Scientific).

The fluorescence of the so-called AGEs was measured, as an indicator of the advanced stages of the Maillard reaction, in a Shimadzu RF-1501 fluorescence spectrophotometer (Kyoto, Japan) at an excitation wavelength of 340 nm and an emission wavelength of 415 nm, according to the method of [Ponger, Ulrich, Bensath and Cerami \(1984\)](#). Samples of native, control heated, and glycated β -Lg and SC were dissolved in 0.1 M sodium phosphate buffer (pH 7.0) to give a final concentration of 1 mg/mL ([Corzo-Martinez et al., 2008](#)).

Browning of control heated SC/ β -lg, β -lg:Gal/Lac and SC:Gal/Lac conjugates (1 mg/mL in double-distilled water) was measured at room temperature by absorbance at 420 nm in a Beckman DU 70 spectrophotometer (Beckman Instruments Inc., Fullerton, CA), as an index of the brown polymers formed in more advanced stages of nonenzymatic browning ([Ting & Rouseff, 1986](#)).

2.4. In vitro gastrointestinal digestion.

All SC and β -lg glycoconjugates, as well as the control heated SC/ β -lg samples were digested *in vitro* by following the simplified procedure described by [Moreno](#),

Mackie and Mills (2005). For the gastric digestion step, glycoconjugates (3 mg) were dissolved in 1 mL of simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5). The pH was adjusted to 2.5 with 1 M HCl if necessary. A solution of 0.32% (w:v) porcine pepsin (EC 3.4.23.1) in SGF (pH 2.5) (Sigma, activity of 3,300 units/mg of protein) was added at an approximately physiological ratio of enzyme to substrate (1:20, w:w). The digestion was performed at 37 °C for 2 h. For the intestinal digestion step, the pH was increased to 7.5 with 40 mM NH₄CO₃ (Panreac, Barcelona, Spain) dropwise to inactivate pepsin, and the following was added to adjust the pH to 6.5 and simulate a duodenal environment: (i) a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate (Sigma) and glycodeoxycholic acid (Sigma), (ii) 1 M CaCl₂ (Panreac), and (iii) 0.25 M Bis-Tris (pH 6.5) (Sigma). Solutions of porcine trypsin (EC 3.4.21.4; 0.05%, w:v, Sigma, type IX-S, activity of 14,300 units/mg of protein) and bovine α-chymotrypsin (EC 3.4.21.1; 0.1%, w:v, Sigma, type I-S, activity of 62 units/mg of protein) in water were prepared and added at approximately physiological protein:trypsin:chymotrypsin ratios [1:(1/400):(1/100) (w:w:w)]. Simulated intestinal digestion of samples was carried out at 37 °C for 15 min. After protein hydrolysis, trypsin and chymotrypsin were inactivated either by heating at 80 °C for 5 min or by adding a solution of Bowman-Birk trypsin–chymotrypsin inhibitor from soybean (Sigma) at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix. Digestions were performed without any derivatization of the sulfhydryl groups of cysteine residues in order to remain as close as possible to physiological conditions.

2.5. Size-exclusion chromatography analysis.

Size-exclusion chromatography (SEC) was carried out under nondenaturing conditions (0.05 M sodium phosphate buffer, pH 7.0, containing 0.15 M NaCl) using a Superdex Peptide HR 10/30 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), on an ÄKTA explorer 100 FPLC system. 500 µL of digested glycoconjugates (1 mg/mL) was applied to the column at room temperature. Elution was achieved in isocratic mode at 0.5 mL/min for 50 min and detection of eluting proteins was performed at 280, 214 and 254 nm. The standard proteins used for calibration were lysozyme (14,400 Da), aprotinin (6,500 Da), insulin B chain (3,500 Da), insulin A chain (2,500 Da) and tryptophan (204 Da). Void volume was determined with blue dextran 2000.

2.6. Bacterial cultures.

Escherichia coli (IATA-CBL2, -CBL8 and -CBM1) strains were isolated from faeces of human subjects, and identified at species level by conventional microbiological methods (colony and cellular morphology, and Gram staining) and by using the API20E system (BioMerieux, Lyon, France) and at strain level by RAPD as described elsewhere ([Sánchez, Nadal, Donat, Ribes-Koninckx, Calabuig & Sanz, 2008](#)). They were grown in Brain-Heart broth and agar (Scharlau), and incubated at 37 °C under aerobic conditions.

2.7. Adhesion assays to mucin.

Crude mucin (Type II, Sigma-Aldrich) was diluted in a phosphate buffered solution (PBS) (pH 7.2) (0.5 mg/mL). An aliquot (0.1 mL) of this solution was loaded into

polycarbonate 96-well plates (Costar, Cambridge, MA, USA) and incubated at 4 °C for 24 h. To remove unbound mucin, the wells were washed twice with 0.1 ml PBS. A fluorescence-based method for the detection of adhesive properties of *E. coli* strains was used (Izquierdo, Medina, Ennahar, Marchioni & Sanz, 2008). Bacteria from 20-hour-old cultures were collected by centrifugation (4,000 x g for 5 minutes at 4 °C), washed twice and resuspended in PBS to reach an optical density of 0.5 ($A_{\lambda 600}$). Suspensions of the different bacterial strains (6.0×10^7 - 8.2×10^8 CFU/mL) were incubated with 75 µmol/L carboxyfluorescein diacetate (CFDA) (C4916, Sigma-Aldrich), at 37 °C for 30 minutes. Then, bacteria were washed twice, resuspended with PBS and split in several sets. Samples were mixed or not with β-Lactoglobulin, sodium caseinate and their glycosylated derivatives (final concentration of 300 µg/mL). Afterwards, 0.1 mL of this cell labelled suspension was loaded into mucin-covered 96-well plates and incubated at 37 °C for 1 hour. Afterwards, the media was removed and wells were washed twice with PBS. Then, 0.2 mL 1% (w/v) sodium dodecyl sulphate (SDS) in 0.1 mol/L NaOH was added to the wells and incubated at 37 °C for 30 min. The mixtures were homogenized by pipetting and the supernatants were transferred to black 96-well plates. The fluorescence was measured in a multiscan fluorometer (Fluoroskan Ascent, Labsystem, Oy, Finland) at λ_{ex} 485 and λ_{em} 538 nm. Negative controls without bacteria were used throughout the experiment.

Adhesion was expressed as the percentage of fluorescence recovered after attachment to mucin relative to the initial fluorescence of the bacterial suspension added to the wells.

2.8. Competitive adhesion assays in the presence of lectins.

The interactions of bacterial suspensions with the following commercially available FITC-labeled lectins (Invitrogen, Paisley, UK) were evaluated: soybean agglutinin (SBA) (L11272), peanut agglutinin (PNA) (L7381), and *Helix pomatia* agglutinin (L11271). Bacterial cells (20 h-old cultures) were harvested, washed three times with PBS (pH 7.2) and then suspended in fresh PBS. Three different sets of samples were prepared; (1) non treated bacterial cells (control), (2) bacterial cells mixed with specific agglutinin (final concentration of 20 µg/ml) for 15 min, and (3) bacterial cells mixed with β-Lactoglobulin, sodium caseinate or their glycosylated derivatives (final concentration of 300 µg/mL) for 15 min and then washed twice with PBS and resuspended in PBS containing the specific agglutinin (20 µg/ml) for additional 15 min. After the incubation period the bacteria were harvested by centrifugation (10,000 x g / 5 min / 4 °C), and recovered in 0.5 mL lysis solution (0.1M NaOH, 1% (w/v) SDS). The fluorescence was measured at λ_{ex} 485 and λ_{em} 510 nm. Negative controls without bacteria were also evaluated.

2.9. Virulence factor genes detection.

DNA for amplification was released from whole bacteria by heating (100 °C/6 min) the bacterial suspensions. Polymerase chain reactions (PCRs) were carried out in a total volume of 15 µL, consisting of 2.5 µL (60 ng) of DNA template, 7.5 of FastStart Taq DNA polymerase (Master Mix I, Roche) and 0.5 µL of each primer at appropriate concentrations and to 15 µL with nuclease free water (Roche) (West, Sprigings, Cassar, Wakeley, Sawyer & Davies, 2007; Table 1). PCR program consisted of 1 cycle of denaturation at 94 °C for 2 min, and by 30 cycles of amplification at 94 °C for 20s, at 55 °C for 20s and at 72 °C for 30s using a LightCycler 480® (Roche) system. Afterwards

the PCR amplification products were further analyzed in a high-resolution 4.5% agarose gel and visualized with ethidium bromide staining.

2.10. Statistical analysis.

Each of the experiments was conducted in triplicate in two independent assays. One-way analysis of variance (ANOVA) and the Tukey's *post hoc* test were applied. Statistical significance was established at $P < 0.05$ for all comparisons. SPSS v.15 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

3. Results and Discussion

3.1. Evolution of the Maillard reaction during the formation of glycoconjugates.

Two different incubation conditions by varying time and temperature were applied for each type of glycoconjugate (β -lg:Gal/Lac and SC:Gal/Lac) and, then, the progress of the Maillard reaction was evaluated by different methods (**Table 2**). Thus, MALDI-TOF and LC/ESI mass spectrometric methods were used to accurately estimate the number of carbohydrate molecules bound to β -lg and SC, respectively, whilst fluorescence and absorbance measurements were carried out to assess the formation of advanced glycation end products (AGEs) and melanoidins (**Table 2**). Considering that the choice of MS instrument largely depends on the nature of the sample to be analysed, among other factors (Yeboah & Yaylayan, 2001), MALDI-TOF-MS analysis allowed a direct and accurate measurement of the glycation degree of a single protein, such as β -lactoglobulin. However, LC/ESI-MS was a more efficient technique to characterize the

glycated SC and to determine the exact number of carbohydrate molecules bound to each individual casein fraction previously separated by RP-LC.

Overall, high level of protein glycation was obtained under all assayed incubation conditions, revealing the efficiency of the Maillard reaction in obtaining multiple saccharides linked to the same carrier (protein). Thus, β -lg was glycated with up to 13 residues of Lac (after 2 days at 60 °C) and 19 residues of Gal (after 2 days at 50 °C), whereas a maximum of 8 molecules of Lac (after 1 day at 60 °C) and 14 of Gal (after 4 hours at 60 °C) were bound to SC. As an example, **Figure 1** shows a typical MALDI-MS spectrum of β -lactoglobulin glycated with Lac for 2 days at 60 °C, as well as three deconvoluted ESI-MS spectra of SC glycated with Lac for 8 hours at 60 °C.

As it was expected, and despite the harshest conditions used for protein lactosylation, Gal was more reactive than Lac to form the glycoconjugates. The order of sugar reactivity in the Maillard reaction is well stated ([Chevalier, Chobert, Mollé & Haertlé, 2001](#); [Nacka, Chobert, Burova, Léonil & Haertlé, 1998](#); [Oliver, Melton & Stanley, 2006c](#); [Corzo-Martinez et al., 2010a](#)) as monosaccharides are more reactive than disaccharides and these more reactive than polysaccharides. Thus, it is known that 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.

On the other hand, a substantial increase in AGEs and melanoidins was observed for each glycoconjugate as the incubation temperature and time increased, indicating the progress of the Maillard reaction (**Table 2**). Thus, within every combination of carbohydrate and protein, two types of glycoconjugates were prepared at different stages of the Maillard reaction, one of them consisted primarily of carbohydrates adducts with a very low content of melanoidins, while those glycoconjugates incubated

under more severe conditions were also susceptible to contain considerable amounts of AGEs and melanoidins.

3.2. Size-exclusion chromatographic (SEC) analysis of fragments derived from the gastrointestinal digestion of the glycoconjugates.

To simulate the physiological conditions, glycoconjugates were subjected to an *in vitro* gastrointestinal digestion model consisting of a first stage of gastric digestion with pepsin for 2 hours at 37 °C and a second stage of duodenal digestion with trypsin/chymotrypsin for 15 minutes at 37 °C.

Due to the complexity of the Maillard reaction, protein aggregation can take place through different mechanisms. Nonetheless, one of the most common is that the corresponding Amadori compound (formed during the initial stages of the Maillard reaction and derived from the re-arrangement of the Schiff base) is readily degraded to reactive (di)carbonyl intermediates, which may act as precursors of aggregate compounds formed during the advanced and final stages of this reaction (Ledl & Schleicher, 1990; Yaylayan & Huyghues-Despointes, 1994). Since the Maillard reaction progressed to advanced stages upon the incubation time and temperature, we have considered the existence of peptide aggregates as a potential factor that could influence the anti-adhesive properties of the produced compounds. Thus, to assess digestibility and size of generated peptides, hydrolysed glycoconjugates were analysed by SEC at pH 7.0 in the presence of 0.15 M NaCl. SC glycoconjugates were more efficiently digested than complexes derived from β -lg, regardless of the employed glycation conditions (**Figure 2**). This result can be attributed to the fact that caseins have a flexible and linear conformation rather than a rigid and compact structure

(Kaminogawa, 2000), facilitating their enzymatic digestion. In contrast, β -lactoglobulin presents a high structural stability at acid pH, having its peptic cleavage sites (hydrophobic or aromatic amino acid side chains) buried inside its characteristic β -barrel structure, forming a strong hydrophobic core and preventing hydrolysis (Reddy, Kella & Kinsella, 1988; Dalgalarondo, Dufour, Chobert, Bertrand-Harb & Haertlé, 1995).

Concerning aggregation levels upon the incubation period, the most notable differences were found following SC glycation with Gal as it was observed a substantial increase in the area of the less retained peaks after incubation under more severe conditions, i.e. 3 days at 50 °C (**Figure 2A**). This behaviour could also be observed for β -lg glycoconjugates (**Figures 2C** and **2D**). The formation of protein aggregates with increasing incubation time and/or temperature may be related to important structural changes derived from extensive cross-linking reactions that occur during the advanced stages of the Maillard reactions.

3.3. Effects of glycated proteins on inhibition of bacterial adhesion.

The effects of β -Lg, SC and their glycoconjugates, after *in vitro* gastrointestinal digestion, on the adhesion percentages of different *E. coli* strains are shown in **Table 3**. *E. coli* strains tested exhibited similar ($P>0.05$) adhesion values to mucin-covered wells. Only native (unglycated) β -Lg and SC caused a significant increase ($P<0.05$) of the adhesion percentages of *E. coli* CBL8 to mucin when compared with controls; however, this effect was not observed for *E. coli* CBL2 and CBM1 strains. Native β -Lg or SC subjected to the harshest experimental conditions applied during the glycation process

386 did not affect differently the adhesion percentages of the *E. coli* strains in comparison to
387 the glycosylated counterparts (*data not shown*).

388 The glycoconjugates of both β -Lg and SC influenced bacterial adhesion percentages
389 as a function of the *E. coli* strain considered. The galactose- or lactose-derived
390 conjugates of β -Lg decreased the adhesion of *E. coli* CBL2, and there were not
391 statistical differences depending on the number of bound galactose molecules for each
392 derivative. Nevertheless, lactose-derived β -lg and SC glycoconjugates incubated under
393 the most severe conditions also decreased, although to a lesser extent, the adhesion of *E.*
394 *coli* CBL2 (**Table 3**). The adhesion of *E. coli* CBM1 was significantly ($P<0.05$)
395 decreased by lactose- and galactose-derived glycoconjugates of β -lg, as well as by
396 galactose-derived glycoconjugates of SC, obtained with the harshest glycation
397 conditions applied (2 days at 60 °C and 50 °C for lactosylated and galactosylated β -lg
398 conjugates, respectively, and 3 days at 50 °C for galactosylated SC conjugates). The
399 products generated during the Maillard reaction did not interfere with the effect of these
400 glycoconjugates on *E. coli* CBL8 adhesion (**Table 3**).

401 Adhesion of pathogenic bacteria to the intestine has been considered as a first step
402 of invasion and infections. However, scarce studies have been done to evaluate the
403 potential properties of dietary carbohydrates or derivatives to inhibit adhesion of
404 potentially pathogenic intestinal bacteria ([Shoaf, Mulvey, Armstrong & Hutkins, 2006](#);
405 [Laparra & Sanz, 2009](#); [Sarabia-Sainz et al., 2009](#).) In this study, adhesion of the *E. coli*
406 strains tested are in the same range as those previously reported for *E. coli* CBL2 (5.1%)
407 by using a classical mucin adhesion assay and different configurations of a human
408 intestinal epithelial cell line (Caco-2) culture ([Laparra & Sanz, 2009](#)). These authors
409 demonstrated significant differences ($P<0.05$) in the adhesion percentages of two *E. coli*
410 strains (CBL2 and CBD10) isolated from faeces, although, both exhibited a preferential

adhesion to intestinal cells. In this study, the inhibition of *E. coli* adhesion by β -Lg or SC glycoconjugates was strain-dependent, suggesting the involvement of different carbohydrate-recognition sites in the interactions between the glycoconjugates and the cell surface molecules of the bacteria.

Factors involved in pathogen adhesion to the intestinal mucosa include lectin-like structures that bind carbohydrates (Ofek, Hasty, Abraham & Sharon, 2000; Shoaf et al., 2006) or glycoconjugate receptor molecules (Ofek et al., 2000). The binding specificity of *E. coli* strains to different defined lectins is shown in **Figure 3A**. *Soybean agglutinin* (SBA) binds specifically to α - and β -N-acetilgalactosamine (galNAc) and galactopiranosil residues, *Helix pomatia agglutinin* (HPA) exhibits affinity for α -N-acetilgalactosamine residues, and *Peanut agglutinin* (PNA) for β -galactose(1-3)galNAc residues. The different *E. coli* strains tested exhibit similar PNA recognition capacity. However, there was a significant difference on the recognition of SBA following the order: CBL2 > CBM1 > CBL8. In addition, *E. coli* CBM1 exhibited the lowest binding capacity to HPA. These results evidenced the abundance of sites recognized by SBA in the *E. coli* strains tested; therefore, SBA was used for competition assays with β -Lg:Gal (1 day/40 °C) or SC:Gal (4 h/60 °C) (**Figure 3B**). These glycoconjugates were chosen because of the higher efficiency in the glycation process, diminishing the appearance of advanced products of the Maillard reaction (**Table 2**). The incubation of both glycoconjugates with the bacterial suspensions caused a significant ($P<0.05$) decrease in SBA binding to *E. coli* CBL2 and CBM1, but did not affect SBA binding to *E. coli* CBL8. The sharp reduction in the SBA binding to *E. coli* CBL2 is concordant with the marked ($P<0.05$) reduction in the adhesion percentages to mucin calculated for this strain in the presence of the glycoconjugates (**Table 3**). In contrast, SBA binding capacity to *E. coli* CBL8 was not affected by the glycoconjugates. This behaviour is in

agreement with the unaltered adhesion percentages of *E. coli* CBL8 to mucin in presence of both compounds tested. Interestingly, both glycoconjugates caused a significant ($P<0.05$) decrease in the SBA binding capacity of *E. coli* CBM1, but it was not accompanied of a significant reduction in its adhesion values (**Table 3**). These results indicate that molecules of a different nature are involved in the adhesion of *E. coli* CBM1 to mucin. Taken together, these results suggest that galactose-reactive lectin like adhesins may be responsible, at least in part, for the recognition of the glycoconjugates tested in the adhesion assays.

It has been reported that several oligosaccharides (firstly, galactooligosaccharides and, secondly, lactulose, inulin and inulin-like) inhibit enteric pathogen adhesion interfering with their recognition binding sites that coat the surface of the gastrointestinal epithelial cells (Shoaf et al., 2006; Kunz & Rudloff, 2008). In this study, the results strongly suggest that both β -Lg:Gal/Lac and SC:Gal/Lac glycoconjugates are recognized by *E. coli* CBL2 clearly interfering with its adhesion to mucin. To the best of our knowledge, these results represent the first evidence of the inhibitory effect of galactosylated proteins, obtained via Maillard reaction, on *in vitro* *E. coli* adhesion after simulated gastrointestinal digestion. However, it has been previously reported that undigested neoglycoconjugates obtained by non-enzymatic lactosylation of serum albumin from different species can reduce *E. coli* adhesion (Ledesma-Osuna et al., 2009; Sarabia-Sainz et al., 2009), and liver and plant lectins adhesion (Aring et al., 1989; Ledesma-Osuna et al., 2008).

The different effects caused by the glycoconjugates on adhesion of the *E. coli* strains tested evidence the heterogeneity of molecules involved in such interactions in this bacterial group. Some of the molecular pathogenic mechanisms of *E. coli* have been characterized, revealing the involvement of complex pathogen-host interactions through

a heterogeneous group of protein(aceous) surface molecules (Fleckenstein, Hardwidge, Munson, Rasko, Sommerfelt & Steinsland, 2010). The involvement of enterotoxigenic invasion protein A (Tia) binding to acidic carbohydrates such as heparan sulfate proteoglycans (Fleckenstein, Holland & Hasty, 2002), among others, has been reported. Proteoglycans composed of disaccharide units, hexosamine (D-glucosamine or D-galactosamine) and uronic acid (D-glucuronic acid or L-iduronic acid) or galactose (keratan sulfate) are also major components in the extracellular matrix, which might constitute binding sites for bacterial adhesion (Sasisekharan & Myette, 2003; Laparra, Lopez-Rubio, Lagaron & Sanz, 2010). Mucins are a family of high molecular weight heavily glycosylated proteins (glycoconjugates), produced by epithelial tissues that are also potential targets for the *E. coli* Tia-mediated adhesion to mucin.

The gene carriage of virulence factors such as lectin like adhesins (fimbriae or pili) can also have an important impact on the ability of *E. coli* strains to colonize the small intestine (Fleckenstein et al., 2010). In this study, the presence of different virulence factor genes in the *E. coli* strains was tested by PCR (**Table 1**) using a normalized DNA concentration (60 ng). The resulting amplification products were resolved in high resolution agarose (4.5%) gel (**Figure 4**). The results revealed that CBL8 and CBM1, but not CBL2, exhibited amplification products for K88, although, all three strains presented amplification products for K99, F41 and heat labile toxin (Hlt).

The gene carriage of F41, but not K88, may explain, at least in part, the differences observed in the inhibitory effect of β -Lg:Gal (1 day/40 °C) or SC:Gal (4 h/60 °C) in *E. coli* CBL2 and CBM1 adhesion. The observation that neoglycoconjugates did not affect *E. coli* CBL8 adhesion could be attributed to the absence of their interaction with the fimbrial adhesin K88, which was not detected in *E. coli* CBL2 (**Figure 4**). Specific associations of the virulence factors, K88/Hlt and K99/F41, have been reported in

porcine *E. coli* ETEC strains (West et al., 2007), which frequently express fimbrial adhesins similar to human *E. coli* ETEC strains (Fleckenstein et al., 2010). Research efforts were made identifying the participation of fimbriae K88 (Sarabia-Sainz et al., 2009; Koh, George, Brözel, Moxley, Francis & Kaushik, 2008) and K99/F41 in the porcine *E. coli* ETEC adhesion to human intestinal embryonic (INT-407) (Koh et al., 2008) or epithelial (Caco-2) cells (Roubos-van den Hil, Nout, Beumer, van der Meulen & Zwietering, 2009).

4. Conclusion

Our results show that gastrointestinal digested bovine β -lg-Gal/Lac and SC-Gal/Lac glycoconjugates may contribute to inhibiting *E. coli* adhesion to mucin. These glycoconjugates showed a strain-dependent effect, suggesting the involvement of different carbohydrate-recognition sites. In this sense, competitive adhesion assays for lectin-like adhesion sites in the *E. coli* strains suggested that galactose-reactive sites may be partly responsible for the recognition of these glycoconjugates. Furthermore, the extent of the Maillard reaction could also have an effect of the anti-adhesive activity of glycoconjugates. Although *in vivo* studies should be conducted, the present findings indicate that production of Maillard-type glycoconjugates, with a high number of attached carbohydrates, is a possible strategy to block pathogen adhesins involved in mucosal colonization and infections.

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Figure Captions.

Figure 1. (A) MALDI-TOF-MS spectrum of β -Lg glyated with Lac at 60 °C for 2 days; and deconvoluted ESI-MS spectra of glyated forms of (B) κ -, (C) α_{s1} -, and (D) β -casein for SC glyated with Lac at 60 °C for 8 hours.

Figure 2. Size-exclusion chromatography profile of the *in vitro* gastrointestinal digested glycoconjugates derived from SC (A and B) and β -Lg (C and D). Elution positions of standard proteins are indicated by arrows: a, lysozyme (Mr 14,400); b, aprotinin (Mr 6,500); c, insulin B chain (Mr 3,500); d, insulin A chain (Mr 2,500); e, tryptophan (Mr 204).

Figure 3. Lectin (soybean agglutinin, SBA; peanut agglutinin, PNA, and *Helix pomatia* agglutinin, HPA) binding to different *E. coli* strains (A) and (B) effect of the different glycoconjugates, after *in vitro* gastrointestinal digestion, on soybean agglutinin binding by different *E. coli* strains. * Indicates statistically significant differences for the same strain.

Figure 4. Virulence factor genes in different *Escherichia coli* strains; fimbrial antigens (K99, F41, K88) and heat labile toxin (Hlt).

Table 1. Oligonucleotide primers used for virulence factor genes amplification.

Target	Oligonucleotide sequence (5'–3')	Working concentration (μ M)	Accession number	Product size (bp)
K88 ^a	ggttcagtgaagtcacatct	20	AJ616236	70
	ccccgtccgcagaagtaac	20		
K99 ^a	gctattagtggtcatggcactgtag	35	M35282	80
	ttgttttcgctaggcagtcatta	35		
F41 ^a	ctgctgattggacggaaggt	30	X14354	88
	ccagtcttccatagccatttaacag	30		
Hlt ^b	ccggcagaggatggttacag	20	K01995	73
	gaatccagggttctctctccaa	20		

^a Fimbrial antigens; ^b Hlt, heat labile toxin.

Table 2. AGE fluorescence, absorbance at 420 nm and number of galactose (Gal) or lactose (Lac) molecules bound to glycated β -lactoglobulin (β -Lg) and sodium caseinate (SC) estimated by mass spectrometry.

Glycoconjugates	Incubation conditions	Number of attached carbohydrate adducts	AGEs (Fluorescence Intensity)	Melanoidins (Absorbance Units)
β -Lg:Gal	40 °C, 1 day	14 ^a	46.0 $\pm$ 2.6 ^c	0.022 $\pm$ 0.002
	50 °C, 2 days	19 ^a	90.5 $\pm$ 0.3	0.229 $\pm$ 0.002
β -Lg:Lac	60 °C, 8 hours	10 ^a	12.6 $\pm$ 0.6	0.004 $\pm$ 0.000
	60 °C, 2 days	13 ^a	74.0 $\pm$ 0.3	0.139 $\pm$ 0.001
SC:Gal	60 °C, 4 hours	14 ^b	17.0 $\pm$ 0.5	0.010 $\pm$ 0.001
	50 °C, 3 days	Not detected	59.5 $\pm$ 0.8	0.101 $\pm$ 0.014
SC:Lac	60 °C, 8 hours	6 ^b	16.4 $\pm$ 1.1	0.006 $\pm$ 0.001
	60 °C, 1 day	8 ^b	46.6 $\pm$ 1.1	0.059 $\pm$ 0.003

^a Average number estimated by MALDI-TOF-MS.

^b Estimated by LC/ESI-MS according to Corzo-Martinez et al. (2010a).

^c Data are average of two independent experiments $\pm$ standard deviation of the mean.

Table 3. Effect of the different glycoconjugates (Gal, galactose and Lac, lactose) of β -lactoglobuline or sodium caseinate, after *in vitro* gastrointestinal digestion, on the adhesion percentages of *E. coli* strains CBL2, CBL8 and CBM1 to mucin. ^{a-c} Different case letters within a row indicate statistically significant ($P<0.05$) differences.

Bacteria	Mucin	β -lactoglobulin				
		Native	Gal, 1d/40 °C	Gal, 2d/50 °C	Lac, 8h/60 °C	Lac, 2d/60 °C
<i>CBL2</i>	5.56 ± 0.31^a	5.55 ± 0.41^a	3.49 ± 0.36^b	3.62 ± 0.59^b	3.67 ± 0.28^b	4.31 ± 0.31^c
<i>CBL8</i>	$5.30 \pm 0.41^{a,b,c}$	6.56 ± 0.38^d	5.19 ± 0.08^a	$5.62 \pm 0.48^{c,d}$	$5.23 \pm 0.23^{a,b}$	$6.07 \pm 0.64^{b,c,d}$
<i>CBM1</i>	$5.09 \pm 0.80^{a,b}$	$4.78 \pm 0.51^{a,b}$	$4.44 \pm 0.58^{b,c}$	3.94 ± 0.19^c	5.20 ± 0.36^a	4.00 ± 0.18^c
	Mucin	Sodium caseinate				
		Native	Gal, 4h/60 °C	Gal, 3d/50 °C	Lac, 8h/60 °C	Lac, 1d/60 °C
<i>CBL2</i>	5.56 ± 0.31^a	6.05 ± 0.41^a	3.48 ± 0.48^b	$4.27 \pm 0.44^{a,b}$	3.63 ± 0.34^b	4.44 ± 0.62^c
<i>CBL8</i>	$5.30 \pm 0.41^{a,b}$	$5.91 \pm 0.75^{a,b}$	6.11 ± 1.07^b	4.95 ± 0.07^a	$5.88 \pm 1.26^{a,b}$	$5.45 \pm 0.35^{a,b}$
<i>CBM1</i>	$5.09 \pm 0.80^{a,b}$	5.62 ± 0.48^b	$5.43 \pm 0.57^{a,b}$	4.21 ± 0.39^c	5.73 ± 0.29^b	$5.05 \pm 0.63^{a,b}$

Figure 1

Figure 1. *Laparra et al.*

Figure 2. *Laparra et al.*

Figure 3. *Laparra et al.*

Figure 4. *Laparra et al.*