



## Functionality of glycomacropeptide glycated with lactose and maltodextrin

Karen Keel,<sup>1\*</sup> Federico M. Harte,<sup>2</sup> Julio Berbejillo,<sup>1</sup> and Tomás López-Pedemonte<sup>1</sup>

<sup>1</sup>Unidad de Ciencia y Tecnología de Lácteos, Universidad Tecnológica del Uruguay, La Paz, Colonia, Uruguay CP 70200

<sup>2</sup>Department of Food Science, The Pennsylvania State University, University Park 16802

### ABSTRACT

The Maillard reaction (MR), under proper environmental conditions, has been used to improve protein functionality. In the present work, 2 high temperatures (50–80°C) and water activity ( $A_w$ ; 0.45–0.67) were used to promote exogenous glycosylation of glycomacropeptide (GMP) while minimizing processing times (0, 8, 24, 48, and 96 h at 50°C; 0, 2, 4, 8, and 24 h at 80°C). Maltodextrin, a polysaccharide commonly used in the food industry as a functional ingredient, was used as a reducing sugar, and compared with lactose, a native milk sugar. The progression of MR was evaluated by tracking changes in molecular weight using SDS-PAGE, the formation of Amadori compounds, and browning. Aqueous glycosylated GMP solutions (5 to 20% wt/vol) were tested for solubility, rheological properties, and foam formation. As expected, MR progression was faster with  $A_w = 0.67$  and 80°C. Glycosylated GMP powders showed no change in their solubility after MR. However, the apparent viscosity ( $\dot{\gamma} = 30 \text{ s}^{-1}$ ) of the 20% wt/vol suspensions exhibited a slight increase when GMP was glycosylated with maltodextrin for 24 h at 80°C, and a 2-log increase when GMP was glycosylated with lactose, with a high browning development in both cases. The foam expansion index of the resuspended glycosylated powders was increased by between 25 and 66% compared with the nonglycosylated powders. Better foam stability (approximately 2 h) and no browning development were observed for GMP glycosylated with maltodextrin for 2 h at  $A_w = 0.67$  and 80°C. The results show that GMP has undergone further glycosylation by means of controlled MR, which improves viscosity and foaming index without negatively affecting solubility. These preliminary studies provide a basis for the future creation of a new ingredient with GMP and reducing sugars.

**Key words:** glycation, glycomacropeptide, Maillard reaction

### INTRODUCTION

During cheesemaking, the enzyme chymosin hydrolyzes  $\kappa$ -CN, increases the casein micelle surface hydrophobicity, and gives rise to curd formation. When  $\kappa$ -CN is hydrolyzed, the following 2 fractions are obtained: the hydrophobic para- $\kappa$ -CN (residue 1–105), which remains buried in the casein micelle, and a hydrophilic peptide (residue 106–169), which is expelled with the sweet whey. This last peptide is known as caseinoglycomacropeptide or glycomacropeptide (GMP) due to the high content of oligosaccharides attached to the peptide chain (approximately 50–60% of the GMP is glycosylated; Kreuz et al., 2009). In this work, the term GMP is used to describe bovine glycomacropeptide, regardless of the native glycosylation level. Glycomacropeptide is highly soluble, stable to thermal processing, and rich in Pro, Glu, Ser, and Thr (Thomä-Worringer et al., 2006). The absence of Trp, Tyr, Phe, or Cys makes GMP an excellent source of protein for phenylketonurics (Thomä-Worringer et al., 2006; Neelima et al., 2013). The molecular mass of GMP varies between 7 to 11 kDa depending on the level of posttranslational glycosylation and phosphorylation (Kreuz et al., 2009; Neelima et al., 2013). Molecular weights ( $M_w$ ) between 14 and 30 kDa have also been reported, and are associated with polymeric forms of GMP (Farías et al., 2012). Kappa-casein natural glycosylation generally occurs on the Thr and Ser residues at the C-terminus, which are part of the GMP. Thr 121, Thr 131, Thr 133, Thr 136, and Thr 142 are accepted as the most important glycosylation sites, but Thr 165, Thr 135, Ser 141, and Ser 142 have also been proposed as potential glycosylation sites (Thoma-Worringer et al., 2006). The glycans can be presented as mono-, di-, tri-, or tetra-saccharides, leading to a great variety of GMP types (Vreeman et al., 1977; Kreuz et al., 2009; Bijl et al., 2014; Jensen et al., 2015).

For both the food and pharmaceutical industries, the Maillard reaction (MR) has proven to be a simple and promising method for the exogenous glycosylation of proteins, resulting in improved technological functionalities, including thermostability, foaming, emulsifying properties, solubility, and stability against changes in

Received February 11, 2022.

Accepted July 7, 2022.

\*Corresponding author: [karen.keel@utec.edu.uy](mailto:karen.keel@utec.edu.uy)

temperature, pH, and ionic strength (Hiller and Lorenzen, 2010; Martínez-Alvarenga et al., 2014; Nooshkam et al., 2018, 2020). Maillard reactions depend largely on water activity ( $A_w$ ) and reach maximum reaction rates at  $A_w$  between 0.6 and 0.8, and drop above or below this range (Labuza, 1970; Roos et al., 2018).

Maillard reactions are initiated by forming an unstable Schiff base through condensing a carbonyl group of a reducing sugar with a free amino group of a protein, which is then rearranged to stable Amadori or Heyns products based on the initial sugar (Martínez-Alvarenga et al., 2014; Liu and Zhong, 2015). A high degree of complexity is observed in the advanced stages of MR, resulting in a range of not well characterized colored nitrogenous polymers and copolymers, known by the generic name of melanoidins, which leads to browning (Martínez-Alvarenga et al., 2014).

Therefore, to use protein-glycan conjugates as food ingredients, it is necessary to arrest MR in the early stages and to avoid the formation of unknown byproducts formed in advanced MR stages (Martínez-Alvarenga et al., 2014; Liu and Zhong, 2015).

Recent studies have focused on the functionality of milk protein-saccharide MR conjugates for a range of technological applications. For example, HTST glycation with lactose and maltodextrin favored the industrial production of high-quality heat-stable whey protein isolate ingredients with reduced browning (Liu and Zhong, 2015), glycosylated sodium caseinate with glucose improved emulsion stability (Da Silva Pinto et al., 2012), and glycosylated  $\beta$ -LG, with glucose and lactose showing greater foaming index and stability (Medrano et al., 2009).

Casal et al. (2005) conducted MR in GMP with lactose as reducing sugar, relatively low temperature (40 and 50°C), intermediate  $A_w$  (0.33 to 0.65), pH between 8 and 11, and extended time of up to 11 d. Furosine, used as an indicator of MR progression, was maximized at 50°C after 5 d of storage at  $A_w = 0.65$  and pH 8 (1.9 mg of furosine/100 mg of GMP), and after 2 d at  $A_w = 0.65$  and pH 11 (1.6 mg/100 mg of GMP). More importantly, improvements in emulsion stability were observed when lactose-GMP was incubated for 2 d at pH 3 and 7 (Moreno et al., 2002).

In the present work, wide and high temperature (50–80°C) and  $A_w$  (0.45–0.67) ranges were used to promote GMP glycation with minimal processing times (up to 96 h). In turn, a polysaccharide commonly used in the food industry as a functional ingredient, maltodextrin, was used as a reducing sugar, which was compared with lactose, a native milk sugar. The overall aim was to use MR to improve the technological properties of GMP so the modified GMP could be used as new functional

ingredients. The effect of MR exposure time, temperature, and  $A_w$  on the solubility, rheology, and foaming properties of glycated GMP were assessed.

## MATERIALS AND METHODS

No human or animal subjects were used, so this analysis did not require approval by an Institutional Animal Care and Use Committee or Institutional Review Board.

### Materials

Glycomacropeptide was obtained from Agropur Ingredients. It was isolated from fresh cheese whey to over 90% purity and spray-dried into powder form. Specifications as provided by the supplier included moisture content  $6.5 \pm 1\%$ , protein content  $93.5 \pm 1.5\%$  db, GMP content  $96.0 \pm 1.5\%$  of total protein, fat  $<0.2\%$ , ash  $6.0 \pm 0.5\%$ , lactose  $0.7 \pm 0.3\%$ , and pH  $6.4 \pm 0.3$ . D-Lactose anhydrous (USP/NF grade) was obtained from MP Biomedicals LLC and Maltodextrin (Globe 019150), with a dextrose equivalent of 14.7%, from Ingredion S.R.L. A micropure-ST system (Thermo Scientific) was used to obtain ultrapure water for all experiments (resistivity of 18.2 M $\Omega$ -cm).

### Preparation of Glycated GMP

Glycomacropeptide and the saccharide (maltodextrin or lactose) were hydrated at a 1:1 mass ratio (100 g of GMP and 100 g of saccharide) in 1 L of 0.1 mol·L<sup>-1</sup> phosphate buffer, pH 7 (mixture of potassium hydrogen phosphate, Mallinckrodt; and potassium dihydrogen phosphate, Merck), stirred for 6 h at room temperature on a magnetic plate set at 300 rpm, frozen to -80°C, and then lyophilized into powders using a LGJ-18 Vikumer lyophilizer. The protein-saccharide powders were kept in desiccators at  $A_w$  of 0.67 or 0.45, using saturated KI or K<sub>2</sub>CO<sub>3</sub> solutions, respectively (Moreno et al., 2002; Corzo-Martínez et al., 2010). To promote MR, the duplicates of powders were exposed to a combination of temperature (50°C for 0, 8, 24, 48, and 96 h; or 80°C for 0, 2, 4, 8, and 24 h) and  $A_w$  (0.45 and 0.67). The same conditions were carried out for glycosylated GMP (**gGMP**), subdivided into gGMP glycosylated with maltodextrin (**gGMP-MD**), gGMP glycosylated with lactose (**gGMP-LAC**), and GMP with no added sugar (**ngGMP**), and sugars alone as negative controls. The time,  $A_w$ , and temperature conditions used to promote MR were selected based on Casal et al. (2005), who glycated GMP successfully at 50°C (between 0.3 and 0.65  $A_w$ ), and Liu and Zhong (2015) who promote MR in

they protein isolates at 80°C and 79% humidity. After incubation, the glycosylated powders and controls were frozen at -20°C before further analysis.

### Evaluation of Glycosylation

**Quantification of Amadori Compounds and Browning.** The estimation of Amadori compounds and browning due to MR was done as described by Savre (2016), with modifications. Briefly, for each of the glycoconjugates, a 1% (wt/vol) solution in ultrapure water was prepared and centrifuged at  $13,800 \times g$  for 10 min at room temperature (Centrifuge Eppendorf 5810R). The absorbance of the supernatant was measured using a spectrophotometer (T70 + UV/vis spectrometer, PG Instruments Ltd.) set at 304 nm to determine the formation of Amadori compounds, and at 420 nm to determine browning.

**Molecular Weight of GMP-Saccharide Conjugates.** Changes in the Mw of gGMP conjugates due to MR were tracked using SDS-PAGE under denaturing conditions, using an Enduro modular vertical gel system (Labnet International Products), and following the method described by Laemmli (1970), with modifications. The electrophoresis was carried out using 15% acrylamide for stacking gel and 5% acrylamide for running gel, and both gels were made with a stock 30% acrylamide-bis solution (Merck). A constant volume of sample (15  $\mu\text{L}$ ) was loaded into the gels. The samples consisted of 8  $\mu\text{L}$  of 1% (wt/vol) glycoconjugate, 22  $\mu\text{L}$  of ultrapure water, and 10  $\mu\text{L}$  of denaturing buffer [62.5  $\text{mmol}\cdot\text{L}^{-1}$  Tris-HCl (Merck), pH 6.8, 25% glycerol (J.T. Baker Fisher Scientific), 2% SDS (Merck), 0.01% bromophenol blue (Merck), 10%  $\beta$ -mercaptoethanol (Applichem Panreac)], and incubated in a dry bath at 100°C for 3 min. The electrophoresis was performed in Tris-Gly running buffer (pH 8.3 at 8°C) for 2 h at 100 V. The protein bands were stained with Coomassie brilliant blue for 30 min and de-stained with a solution of 40% (vol/vol) methanol (Carlos Erba), 10% (vol/vol) acetic acid (Merck), and 45% (vol/vol) distilled water. The Mw of the proteins and conjugates was estimated using an Accuruler RGB pre-stained protein ladder (Maestrogen Inc.).

### Evaluation of Technological Properties

Two sets of solutions were prepared with ngGMP and gGMP. To keep the GMP concentration the same, the first set of solutions was prepared as follows: ngGMP was reconstituted at 5% (wt/vol) in ultrapure water, whereas the gGMP was reconstituted at 10% (wt/vol) in ultrapure water. The second set of solutions were prepared as follows: ngGMP at 20% (wt/vol) in ultra-

pure water and gGMP at 40% (wt/vol) in ultrapure water. All solutions were vortexed twice at 3,000 rpm for 30 s, and kept in a refrigerator at 4°C overnight. Before further evaluation, the samples were stabilized at 20°C, vortexed at 3,000 rpm for 30 s, and centrifuged at  $3,000 \times g$  for 10 min (Centrifuge Eppendorf 5810R) at 20°C.

**Solubility.** Solubility was evaluated according to Sikand et al. (2011), with modifications. The ngGMP and the gGMP were reconstituted in 5% and 10% (wt/vol) ultrapure water solutions, respectively. An aliquot (1  $\text{g} \pm 0.1$  mg) of the original sample, before centrifugation, and the supernatant, after centrifugation at  $700 \times g$  for 10 min at 20°C, were dried at 102°C until a constant weight was achieved (approximately 3 h). The relative solubility of the sample was calculated as the wt/wt ratio between the supernatant and the suspension before centrifugation.

**Flow Properties.** Flow curves (shear strain rate vs. shear stress) on 1.6 mL of liquid samples were done at a 1 to 100  $\text{s}^{-1}$  shear rate and 20°C, using MCR 302 rotational rheometer (Anton Paar GmH), equipped with a cone plate geometry (CP60-1/TG, 60 mm diameter, 1° angle, 120  $\mu\text{m}$  truncation). The flow curves were modeled using the following power law model, appropriate for shear-thinning, time-independent liquids, according to Corzo-Martínez et al. (2010):

$$\tau = K \times \dot{\gamma}^n,$$

where  $\tau$  = stress (Pa),  $K$  = consistency coefficient ( $\text{Pa}\cdot\text{s}^n$ ),  $\dot{\gamma}$  = shear rate ( $\text{s}^{-1}$ ), and  $n$  = the flow behavior index. For the special case of Newtonian liquids ( $n = 1$ ), the consistency coefficient  $K$  is the Newtonian viscosity, whereas, in shear-thinning liquids,  $0 < n < 1$ . Apparent viscosity at a  $\dot{\gamma} = 30 \text{ s}^{-1}$  was used to compare treatment samples when non-Newtonian behavior was observed.

**Foam Properties.** Fifteen milliliters of ngGMP (5% wt/vol in ultrapure water) and gGMP (10% wt/vol in ultrapure water) solutions were placed in 50 mL centrifuge tubes, vortexed at 3,000 rpm for 30 s, and placed in a rack for observation. A continuous video recording of the tubes was done for 40 min. The foam volumes were recorded from 0 to 6 min every 30 s, and from 6 to 40 min every 1 min. To evaluate the foam stability, the foam volume of each sample was measured at time 0 and 18 min. Foam expansion index (**FEI**) was calculated as follows, according to Corzo-Martínez et al. (2010):

$$\text{FEI}(\%) = \frac{V_t}{V_0} \times 100,$$

where  $V_t$  = foam volume formed at time  $t$ , and  $V_0$  = liquid initial volume.

### Experimental Design and Statistical Analysis

Each glycoconjugate, obtained from a set temperature condition (50°C or 80°C) and  $A_w$  (0.45 and 0.67), was analyzed as a completely randomized experiment with time as the fixed treatment effect and 2 replicates. In cases where the ANOVA showed significant treatment effect, multiple means comparisons were done using Tukey's test ( $P < 0.05$ ). The data were analyzed using Infostat (version 9-29-2020, Universidad Nacional de Córdoba).

## RESULTS AND DISCUSSION

Glycomacropeptide further glycosylated via MR is a potential technological solution for the manufacturing of functional protein ingredients. The native level of glycosylation of GMP is variable, given its origin and processing. The heating of the milk or whey before the isolation of GMP influences the extent of glycosylation in isolated GMP, with more severe heating producing GMP with less glycosylation (Neelima et al., 2013). We evaluated the functional properties of GMP glycosylation with relevant polysaccharide ingredients (e.g., maltodextrin and lactose) via MR.

### Evaluation of Glycosylation

**Quantification of Amadori Compounds and Browning.** As expected, ngGMP (GMP only) did not exhibit a significant increase in the development of Amadori compounds and browning, regardless of MR exposure temperature,  $A_w$ , and time (Figures 1 and 2). The ngGMP and sugar controls (lactose and maltodextrin only) did not show significant color changes even after the most severe MR conditions (i.e., 24 h at 80°C; Figure 3).

As the exposure time increased and the MR progressed, Amadori compounds were detected, and increased browning was observed in all gGMP samples (Figure 1). The effect of increased MR exposure time on the formation of Amadori compounds was significant for all gGMP. As expected, in powder samples held at 80°C, MR proceeded at a higher rate for both glycosylated lactose and maltodextrin. For gGMP-MD (GMP glycosylated with maltodextrin), a significant increase in Amadori compounds was obtained after only 2 h of MR at both 0.45 and 0.67  $A_w$ , whereas for gGMP-LAC (GMP glycosylated with lactose), significant differences were observed after 4 h of MR at  $A_w = 0.45$ , and after 24 h at  $A_w = 0.67$ . At 50°C, the development of Ama-

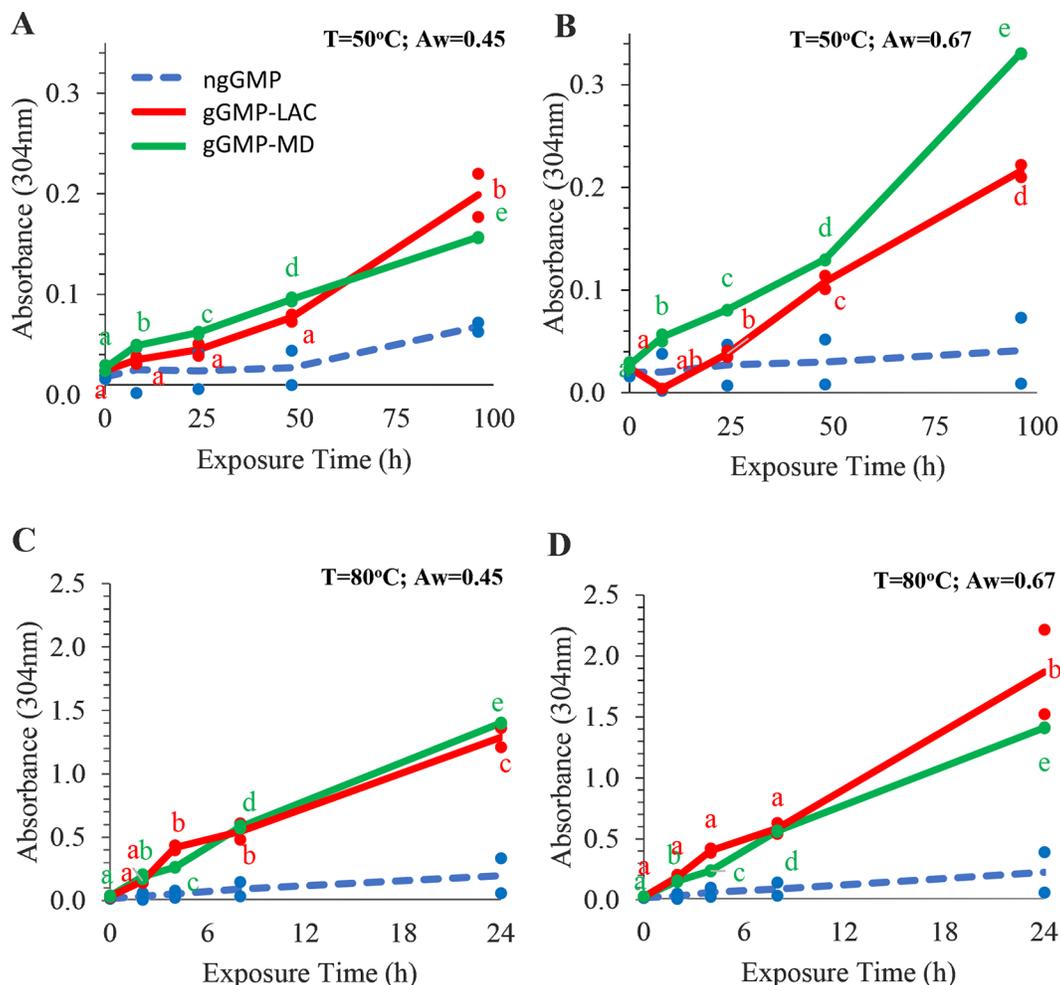
dori compounds was observed at longer exposure times, for gGMP-MD after 8 h of MR and, for gGMP-LAC, after 48 h of exposure at  $A_w = 0.67$  and 96 h at  $A_w = 0.45$ .

Little browning was observed after the glycosylation of GMP at 50°C with lactose and maltodextrin at both  $A_w$ , with maximum absorbance values after 96 h of MR that were similar to those obtained after 8 h at 80°C, and at least 1-log cycle lower than those obtained when the powders were exposed to MR at 80°C for 24 h (Figure 2). Despite obtaining statistically significant differences in gGMP-MD exposed to MR conditions at 50°C for 48 h at  $A_w = 0.45$ , and 96 h at  $A_w = 0.67$ , differences were not relevant as the absorbances at 420 nm were very low and browning was not evident to the naked eye (Figure 3). Samples probably underwent only early stages of the MR, where the development of Amadori compounds did not result in browning.

Browning was perceptible in gGMP-MD exposed to 80°C at both  $A_w$ , and was greater in gGMP-LAC at both temperatures and  $A_w$ , suggesting more advanced stages of MR (Figure 3). After 24 h of exposure at 80°C, statistically significant differences were observed for both gGMP, regardless of  $A_w$ . However, the browning of gGMP-LAC at 0.67  $A_w$  ( $0.493 \pm 0.029$ ) was 2.6 times higher than that of gGMP-MD under the same conditions ( $0.188 \pm 0.054$ ), and almost 4.0 times higher than that of both gGMP at 0.45  $A_w$  ( $0.132 \pm 0.010$  and  $0.112 \pm 0.010$ , respectively). Powders of gGMP-MD did not reach advanced stages of browning, with intermediate products exhibiting only a pale-yellow color, and strong absorption in the UV region due to the absence of dicarbonyls (Abd El-Salam and El-Shibiny, 2018). The formation of Amadori compounds and the development of browning indicated that the fastest progress of MR occurred in GMP glycosylated at 80°C. A higher number of Amadori compounds was produced in gGMP-MD, but with a lower development of browning at higher exposure times.

This work focused on the early to intermediate stages of the MR, where an increase in Amadori compounds was observed, followed by an increase in browning. Reduction stages of Amadori compounds were not reached, such as those observed by Casal et al. (2005) after 5 d at 50°C and pH = 8, or 9 d at pH = 11. Amadori compounds and browning results indicating the progression of MR were consistent with the electrophoresis results, which suggested the formation of GMP polymers.

**Molecular Weight of GMP-Saccharide Conjugates.** To evaluate the change in Mw of GMP due to MR glycosylation, reducing SDS-PAGE was done. In all electrophoresis runs, the characteristic GMP band was observed between 10 and 17 kDa (Figure 4). For gGMP obtained at 50°C at both  $A_w$ , a small increase



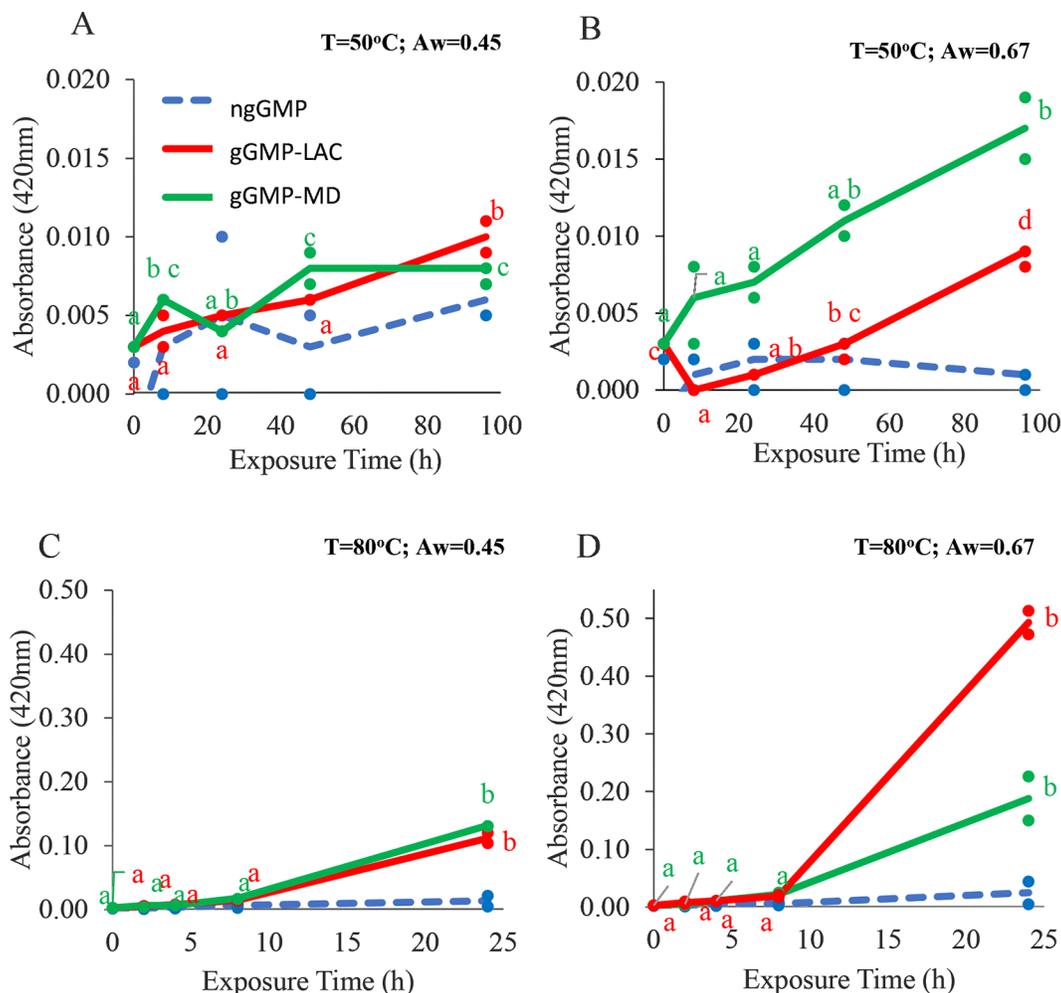
**Figure 1.** Amadori compound formation (measured as absorbance at 304 nm) in samples of glycomacropeptide (GMP) with no added sugar (ngGMP), GMP glycosylated with maltodextrin (gGMP-MD), and GMP glycosylated with lactose (gGMP-LAC), at temperature ( $T$ ) = 50°C and water activity ( $A_w$ ) = 0.45 (A), at 50°C and  $A_w$  = 0.67 (B), at 80°C and  $A_w$  = 0.45 (C), and at 80°C and  $A_w$  = 0.67 (D). Different letters (a–e) indicate significant differences within each GMP type using Tukey-Kramer ( $P < 0.05$ ).

in Mw was observed for gGMP-LAC (from 17 to 25 kDa) as exposure time increased, but no change in Mw of gGMP-MD was observed regardless of exposure time. This could be due to the formation of a variety of compounds generated through reactions of cyclization, dehydration, retroaldolization, enolization, oxidation, fragmentation, acid hydrolysis, isomerization, rearrangement, free radical reactions, and further condensation occurring, leading to the formation of a large number of poorly characterized compounds (Abd El-Salam and El-Shibiny, 2018).

When MR was produced at 80°C in both  $A_w$ , diffuse bands of higher Mw were observed in both gGMP-LAC and gGMP-MD. These bands had approximately 35 kDa after 8 h of MR, and approximately 48, 67, and 75 kDa after 24 h of exposure in the gGMP-LAC samples. In contrast, gGMP-MD showed 1 high Mw band at

approximately 35 kDa, but only after exposure to MR for 24 h at  $A_w$  = 0.67, and several bands (approximately 48, 67, and 75 kDa) after 24 h of exposure at  $A_w$  = 0.45. The wide range of Mw observed (~15 to 75 kDa) was likely due to the generation of heterogeneous MR products, the formation of gGMP aggregates, or covalently linked degradation products; additionally, this was seen to some extent with ngGMP. The presence of high Mw bands associated with the appearance of brown pigments may be due to the generation of melanoidins (Medrano et al., 2009), as well as heterogeneous, multimeric, or lysinoalanine crosslinking products (Zavala Pope, 2009).

Surprisingly, ngGMP exhibited an additional band with Mw between 28 to 35 kDa and also the shadow of a band at ~50 kDa after MR at 80°C for 24 h. This may be explained by limited MR catalyzed by sac-



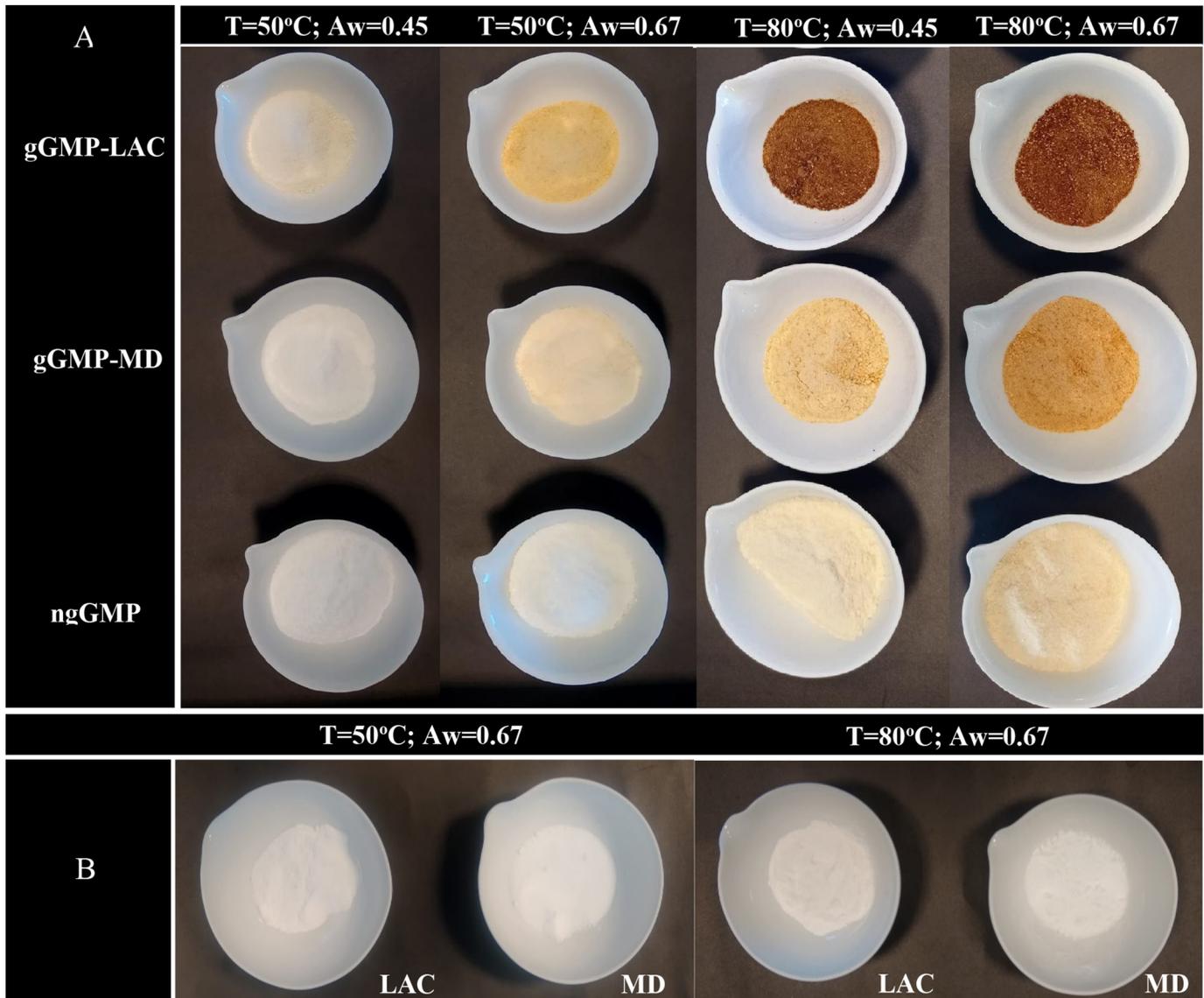
**Figure 2.** Browning (measured as absorbance at 420 nm) in samples of glycomacropeptide (GMP) with no added sugar (ngGMP), GMP glycosylated with maltodextrin (gGMP-MD), and GMP glycosylated with lactose (gGMP-LAC), at T (temperature) = 50°C and water activity ( $A_w$ ) = 0.45 (A), at 50°C and  $A_w$  = 0.67 (B), at 80°C and  $A_w$  = 0.45 (C) and at 80°C and  $A_w$  = 0.67 (D). Different letters (a–d) indicate significant differences within each GMP type using Tukey-Kramer ( $P < 0.05$ ).

charides bound to GMP or small residual lactose left in the GMP powders. The Mw distributions observed in the gels indicate that the degree of glycation was different for each sugar, which was consistent with results reported about  $\beta$ -LG and sodium caseinates, where disaccharides had a greater ability to glycosylate by MR than polysaccharides (Chevalier et al., 2001; Corzo-Martínez et al., 2010).

At a mass ratio of 1:1, the approximate molar ratio of GMP:maltodextrin and GMP:lactose was approximately 1:10 and 1:20, respectively. Glycomacropeptide contains, at most, 3 Lys and 5 Thr available residues, and the saturation with both sugars could potentially lead to browning due to caramelization at high temperature (e.g., 80°C). This was not the case, as both lactose-only and maltodextrin-only controls did not exhibit browning when subjected to the most extreme

temperature and time conditions in these experiments (i.e., 80°C for 24 h; Figure 3).

The concentration of Amadori compounds, the browning development, and the observed protein bands in SDS-PAGE indicated that MR occurred at a faster rate in GMP glycosylated at 80°C. At this temperature, products corresponding to both early and advanced stages of MR were detected at different exposure times. When GMP was glycosylated at 50°C, only products corresponding to the early stages of MR were evident. We were not able to elucidate whether lactose had a faster or slower rate of reaction versus maltodextrin, as the 2 sugars were added in excess amounts versus GMP. Nevertheless, a higher number of Amadori compounds were present in gGMP-MD, with little development of brown pigments, even at longer exposure times.



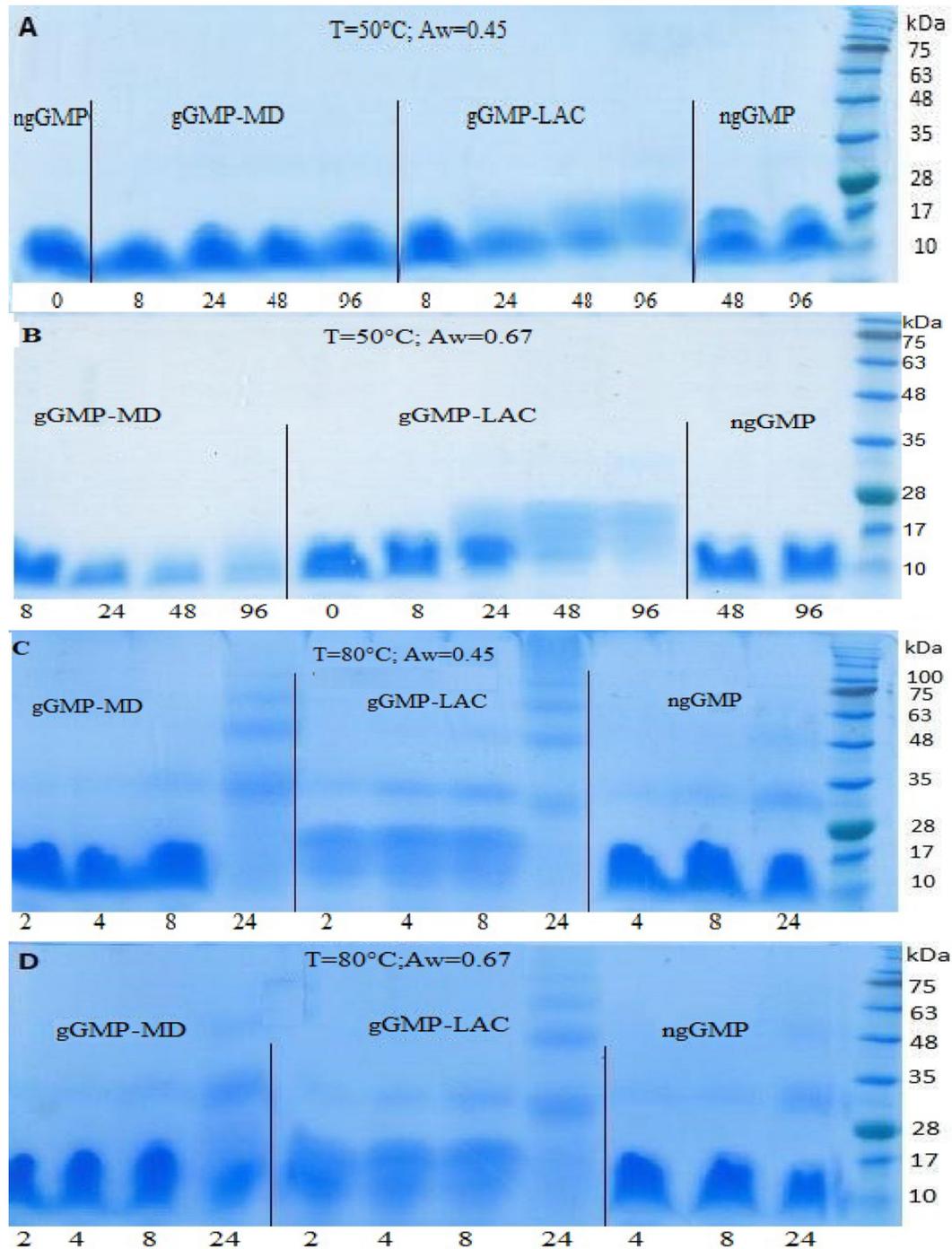
**Figure 3.** Images of the powders exposed to temperature ( $T$ ) = 80°C for 24 h and 50°C for 96 h. Glycomacropeptide glycated with maltodextrin (gGMP-MD) or lactose (gGMP-LAC), and glycomacropeptide with no sugar added (ngGMP) controls (A). Sugar controls (B).  $A_w$  = water activity.

### Evaluation of Technological Properties

**Solubility.** The variation in solubility caused by MR on GMP was studied. The results showed the high solubility of ngGMP was not affected by MR exposure time for controls, and for gGMP/MD and gGMP/LAC (data not shown). These results suggest that GMP could be incubated with both lactose and maltodextrin, at high temperatures (e.g., 80°C) for sufficient periods of time, to obtain highly glycosylated forms without affecting overall solubility. Similar results were found in the glycation of GMP with lactose at 40°C (Moreno et al., 2002). Milk proteins used in food products are

generally required to have high levels of solubility to facilitate expression of desired functionality including gelation, aeration, water binding, foaming, and emulsification.

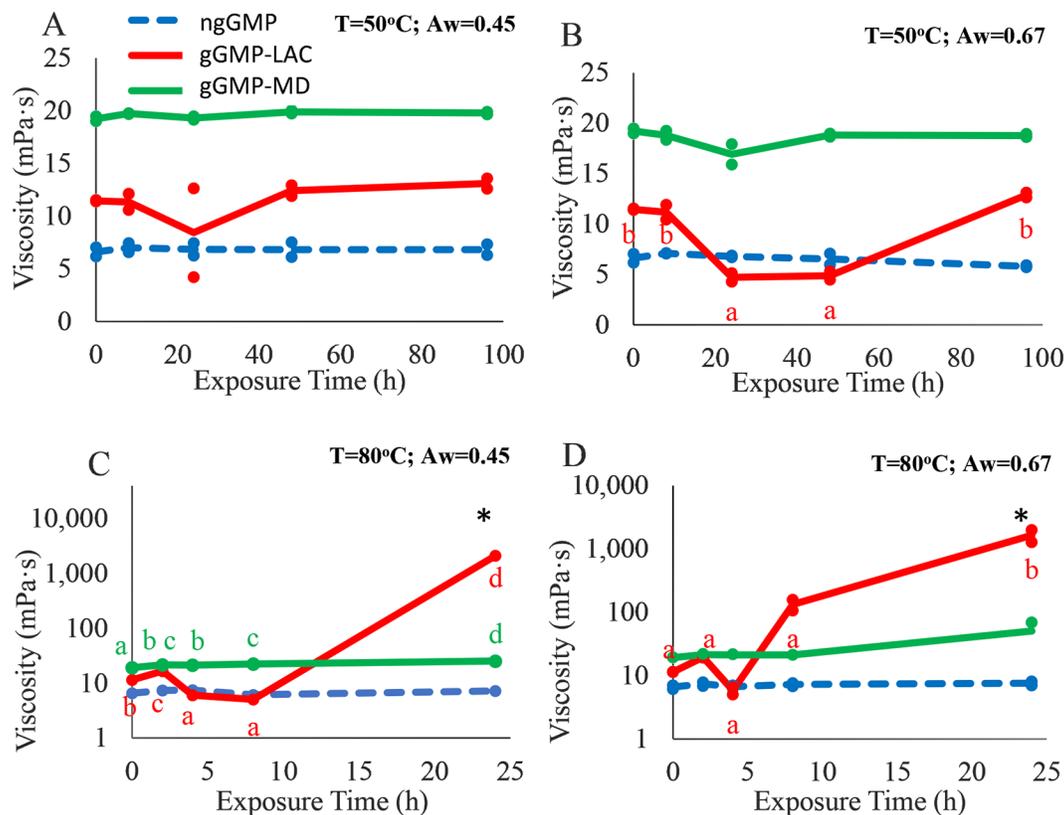
However, there is no general rule about the solubility of glycosylated aggregates after MR, as conflicting reports found reductions in the solubility in glycosylated proteins with secondary and tertiary structure, including ovalbumin (Aoki et al., 2001), shellfish muscle proteins (Katayama et al., 2002), myofibrillar protein (Sato et al., 2000), myosin (Tanabe and Saeki, 2001), whey protein isolate, and  $\beta$ -LG (Medrano et al., 2009; Martínez-Alvarenga et al., 2014). This lack



**Figure 4.** Sodium dodecyl sulfate-PAGE of glycomacropeptide powders not glycosylated (ngGMP), or glycosylated with maltodextrin (gGMP-MD) or lactose (gGMP-LAC) by Maillard reaction. T and  $A_w$  are the temperature and water activity during the Maillard reactions. The numbers at the bottom of each gel are the hours of exposure to Maillard reactions. The last line to the right of each gel is the molecular weight standard, Accruler RGB pre-stained protein ladder (Maestrogen Inc.).

of consistency in previous experimental results emphasizes the need for further understanding of the effect of MR on the technological properties of glycosylated food proteins.

**Flow Properties.** The flow properties of all reconstituted gGMP were evaluated after dispersion of the powders in water at 5% and 20% wt/vol concentration. In all the solutions prepared at 5% wt/vol, Newtonian



**Figure 5.** Viscosity of 20% solution in samples of glycomacropeptide (GMP) nonglycosylated, incubated without saccharide (ngGMP), GMP incubated with maltodextrin (gGMP-MD), and GMP incubated with lactose (gGMP-LAC), at temperature ( $T$ ) = 50°C and water activity ( $A_w$ ) = 0.45 for 0 to 96 h (A), at 50°C and  $A_w$  = 0.67 for 0 to 96 h (B), at 80°C and  $A_w$  = 0.45 for 0 to 24 h (C), and at 80°C and  $A_w$  = 0.67 for 0 to 24 h (D). Different letters (a–d) indicate significant differences within each GMP type using Tukey-Kramer ( $P < 0.05$ ). \*Shear-thinning behavior, apparent viscosity at  $\dot{\gamma} = 30 \text{ s}^{-1}$ .

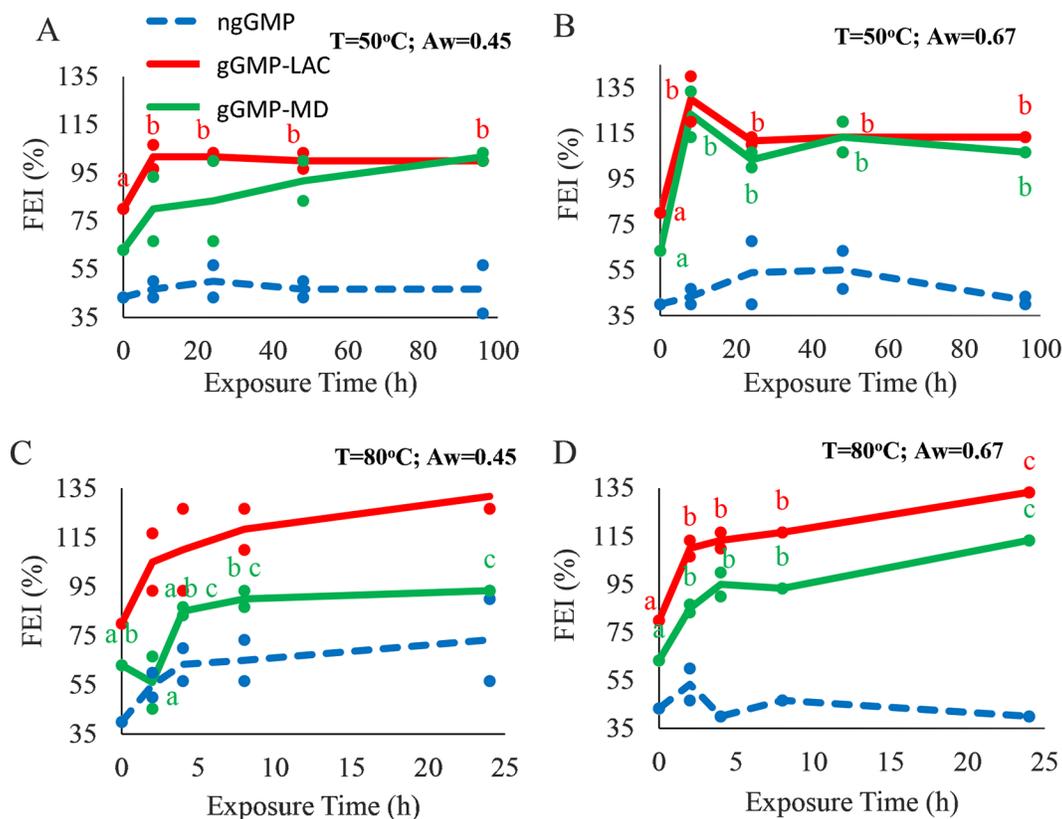
flow characteristics were observed and the MR exposure time did not significantly affect the viscosity of gGMP for the most part. Statistically significant differences were only found for gGMP-LAC at 24 h of MR exposure at 80°C in both  $A_w$ , and the viscosity only changed from 1.8 to 2.6 mPa·s for  $A_w$  = 0.45 and from 1.8 to 2.8 mPa·s for  $A_w$  = 0.67 (data not shown).

For the solutions containing 20% wt/vol concentration, just adding sugars before MR increased the viscosity of the system. In gGMP solutions with both sugars exposed to MR at 50°C, no significant changes in viscosity were observed with increased MR exposure time (Figure 5). However, for gGMP-LAC, a slight reduction in viscosity was observed in 20% solutions after the first hour of exposure to MR (Figure 5), possibly due to the depolymerization or deamination MR intermediates. Glycosylated GMP prepared at 80°C showed strong increase in viscosity and a transition to non-Newtonian behavior as MR progressed. This increase in viscosity was observed in gGMP-LAC at both  $A_w$ , and to a lesser extent in gGMP-MD at  $A_w$  = 0.67 (Figure 5). Additionally, gGMP-LAC exposed to 80°C exhibited

shear-thinning behavior and a strong increase (approximately 2-log) in apparent viscosity ( $\dot{\gamma} = 30 \text{ s}^{-1}$ ) after 8 h of MR exposure at  $A_w$  = 0.67. In other studies, a similar trend was found in the viscosity of caseinate protein when glycosylated with dextran (Hiller and Lorenzen, 2010). This increased viscosity of gGMP at 80°C may be the result of polymerization, as discussed before.

A positive correlation was also found between the viscosity of gGMP solutions (20% wt/vol) and the browning measured as absorbance at 420 nm ( $r = 0.86$ ;  $P < 0.01$ ), and viscosity and presence of Amadori compounds ( $r = 0.66$ ;  $P < 0.01$ ).

**Foam Properties.** All powders were suspended in water and vortexed to evaluate FEI and foam stability. The simple addition of highly functional sugars, without exposure to temperatures or  $A_w$  of the test, increased the foaming properties of GMP, due to a concentration effect that increases the general viscosity of the system. A marked increase in FEI was observed in all gGMP in the early stages of MR (2 h at 80°C and 8 h at 50°C), before the extensive darkening associated



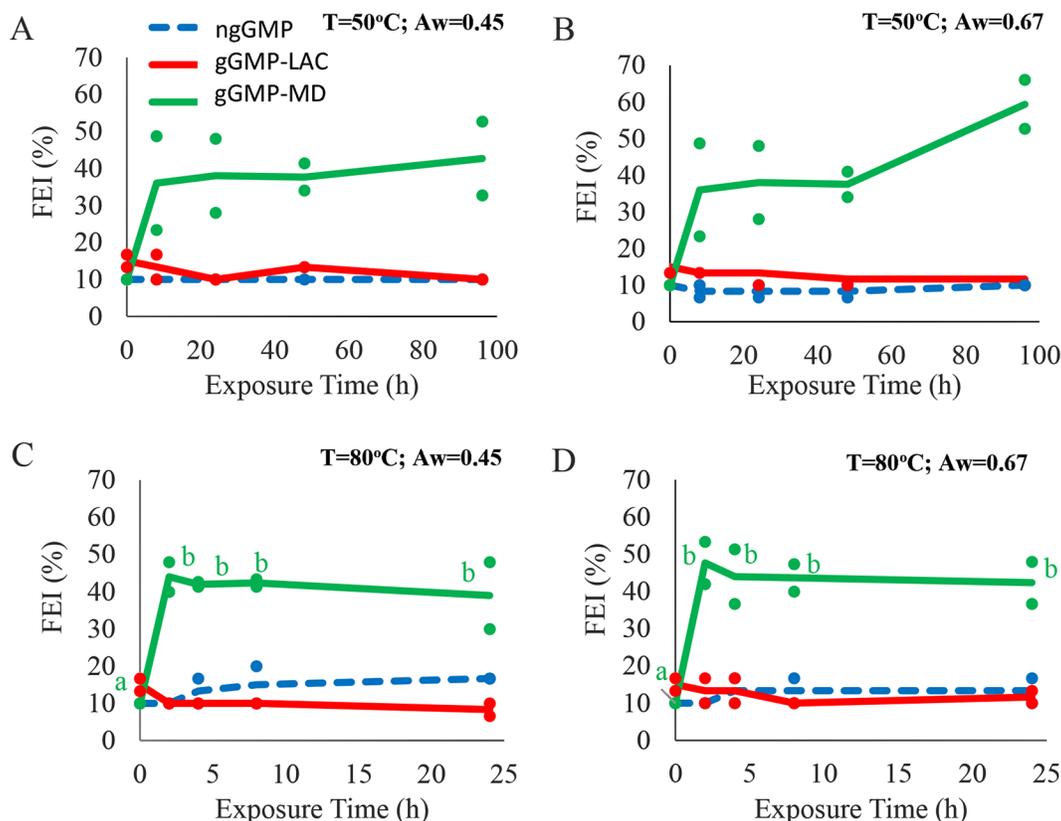
**Figure 6.** Foam expansion index (FEI) at time 0 min in samples of glycomacropeptide (GMP) incubated without saccharide (ngGMP), GMP incubated with maltodextrin (gGMP-MD), and GMP incubated with lactose (gGMP-LAC), at temperature ( $T$ ) =  $50^{\circ}\text{C}$  and water activity ( $A_w$ ) = 0.45 for 0 to 96 h (A), at  $50^{\circ}\text{C}$  and  $A_w = 0.67$  for 0 to 96 h (B), at  $80^{\circ}\text{C}$  and  $A_w = 0.45$  for 0 to 24 h (C), and at  $80^{\circ}\text{C}$  and  $A_w = 0.67$  for 0 to 24 h (D). Different letters (a-c) indicate significant differences within each GMP type using Tukey-Kramer ( $P < 0.05$ ).

with advanced MR. For example, after 2 h at  $80^{\circ}\text{C}$  and  $A_w = 0.67$ , the FEI of gGMP-MD increased from 63 to 85% ( $P < 0.05$ ). For gGMP-LAC, the FEI increased from 80 to 110% ( $P < 0.05$ ). Similarly, after 8 h at  $50^{\circ}\text{C}$  and  $A_w = 0.67$ , the FEI further increased to 123% for gGMP-MD and 130% for gGMP-LAC ( $P < 0.05$ ; Figure 6). This increase in FEI continued to be observed in the following stages for gGMP exposed at  $80^{\circ}\text{C}$ , but not at  $50^{\circ}\text{C}$ . The maximum FEI (136%) was observed for gGMP-LAC after 24 h of MR exposure at  $80^{\circ}\text{C}$  and  $A_w = 0.67$ . These results were consistent with those obtained for SDS-PAGE and viscosity, where products with higher Mw and viscosities had higher FEI.

To study the stability of the foams obtained from reconstituted ngGMP and gGMP powders, the foams were monitored for 18 min after their formation (Figure 7). Foam made with gGMP-LAC rapidly collapsed to levels similar to ngGMP. In contrast, the foams made with gGMP-MD persisted for around 1 h. The FEI obtained with glycosylates at  $80^{\circ}\text{C}$  showed values greater than 40% at 18 min in foams formed with gGMP-MD, and close to 10% with ngGMP and gGMP-LAC. An improvement in foaming stability was observed for

gGMP-MD exposed to MR at  $80^{\circ}\text{C}$  for only 2 h ( $P < 0.01$ ), where Amadori products with no browning were observed (Figures 1 and 2). The gGMP samples exposed to MR at  $50^{\circ}\text{C}$  produced a similar, but not significant trend, due to the high dispersion of the observations.

The formation of a foam requires the participation of a surfactant capable of migrating to the air/water interface to lower surface tension. Native nonglycosylated GMP has better foaming ability than native gGMP. The combination of hydrophilic and electrostatic effects prevents the adsorption of native gGMP molecules at the air/water interface (Kreuz et al., 2009). In contrast, native nonglycosylated GMP molecules form a stable network at the interface (Jauregui-Rincón et al., 2019). However, in milk proteins glycosylated through MR, greater stability of the elaborated foams has been observed (Medrano et al., 2009; Báez et al., 2013). Several mechanisms have been proposed to explain this: the formation of thick, viscoelastic layers of MR products at air/liquid interfaces (Fechner et al., 2007), the increase in viscosity of MR product solutions, and steric protection against aggregation and coalescence of branched-chain polysaccharides (Ganzevles et al.,



**Figure 7.** Foam expansion index (FEI) at time 18 min in samples of glycomacropeptide (GMP) not glycosylated, incubated without saccharide (ngGMP), GMP incubated with maltodextrin (gGMP-MD), and GMP incubated with lactose (gGMP-LAC), at temperature ( $T$ ) = 50°C and water activity ( $A_w$ ) = 0.45 for 0 to 96 h (A), at 50°C and  $A_w$  = 0.67 for 0 to 96 h (B), at 80°C and  $A_w$  = 0.45 for 0 to 24 h (C), and at 80°C and  $A_w$  = 0.67 for 0 to 24 h (D). Different letters (a,b) indicate significant differences within each GMP type using Tukey-Kramer ( $P < 0.05$ ).

2006), and increased protein hydrophilicity (Fechner et al., 2007). Increased stability of protein foams was reported for  $\beta$ -LG glycosylated with glucose and lactose (Chevalier et al., 2001; French et al., 2002; Medrano et al., 2009), and total milk protein glycosylated with glucose, lactose, pectin, and dextran (Hiller and Lorenzen, 2010).

The glycosylated samples that exhibited a strong increase in apparent viscosity, and a transition from Newtonian to shear-thinning behavior, also showed the appearance of several larger Mw protein aggregates in SDS-PAGE (Figure 4). Similar results were reported for glycosylated sodium caseinate with glucose and lactose (Hiller and Lorenzen, 2010).

A positive correlation was also found between the viscosity of gGMP solutions (20% wt/vol) and the browning measured as absorbance at 420 nm, viscosity and presence of Amadori compounds, and viscosity and FEI at zero time for gGMP-LAC (Table 1).

Glycosylation increased the Mw of GMP and probably modified its net charge and charge distribution. Over the course of the exposure time, a diverse popula-

tion of molecules at different MR stages was detected. In further studies, zeta-potential tests could be carried out to understand the charge change, as well as hydrophobicity tests and mass spectrometry to further characterize the MR products.

**Table 1.** Pearson correlation coefficients between absorbance at 420 and 304 nm, viscosity, and foam expansion index (FEI) at 0 min<sup>1</sup>

Item <sup>2</sup>	Absorbance (304 nm)	Viscosity	FEI
GMP-LAC			
Absorbance (420 nm)	0.96**	0.86**	0.50**
Absorbance (304 nm)		0.83**	0.59**
Viscosity			0.50*
GMP-MD			
Absorbance (420 nm)	0.80**	0.86**	0.31*
Absorbance (304 nm)		0.66**	0.31*
Viscosity			—

<sup>1</sup>Only significant correlations are shown.

<sup>2</sup>GMP-LAC = glycomacropeptide glycosylated with lactose; GMP-MD = glycomacropeptide glycosylated with maltodextrin.

\* $P \leq 0.05$ ; \*\* $P \leq 0.001$ .

## CONCLUSIONS

Glycosylated fractions with equal solubility to the initial protein, and higher FEI and viscosity were observed when GMP was exposed to MR in the presence of lactose and maltodextrin. Glycosylation increased the Mw of GMP, and probably its net charge and charge distribution. The increase in viscosity was directly proportional to the increase in the foam expansion in the foam index and the presence of protein aggregates. These results provide a basis for the future creation of a new ingredient with GMP and sugars that will be useful for the food industry.

## ACKNOWLEDGMENTS

This study received no external funding. We thank Andrea Garay for the statistical studies carried out in this research (Unidad de Ciencia y Tecnología de Lácteos, Universidad Tecnológica del Uruguay, La Paz, Colonia, Uruguay). Likewise, we thank Lorena Niddegger (Unidad de Ciencia y Tecnología de Lácteos, Universidad Tecnológica del Uruguay) for her collaboration in the experimental activities. The authors have not stated any conflicts of interest.

## REFERENCES

- Abd El-Salam, M. H., and S. El-Shibiny. 2018. Glycation of whey proteins: Technological and nutritional implications. *Int. J. Biol. Macromol.* 112:83–92. <https://doi.org/10.1016/j.ijbiomac.2018.01.114>.
- Aoki, T., Y. Hiidome, Y. Sugimoto, H. R. Ibrahim, and Y. Kato. 2001. Modification of ovalbumin with oligogalacturonic acids through the Maillard reaction. *Food Res. Int.* 34:127–132. [https://doi.org/10.1016/S0963-9969\(00\)00140-X](https://doi.org/10.1016/S0963-9969(00)00140-X).
- Báez, G. D., P. A. Busti, R. Verdini, and N. J. Delorenzi. 2013. Glycation of heat-treated  $\beta$ -lactoglobulin: Effects on foaming properties. *Food Res. Int.* 54:902–909. <https://doi.org/10.1016/j.foodres.2013.08.013>.
- Bijl, E., R. de Vries, H. van Valenberg, T. Huppertz, and T. van Hooijdonk. 2014. Factors influencing casein micelle size in milk of individual cows: Genetic variants and glycosylation of  $\kappa$ -casein. *Int. Dairy J.* 34:135–141. <https://doi.org/10.1016/j.idairyj.2013.08.001>.
- Casal, E., N. Corzo, F. J. Moreno, and A. Olano. 2005. Glycation of caseinmacropeptide. *Food Chem.* 92:33–36. <https://doi.org/10.1016/j.foodchem.2004.07.005>.
- Chevalier, F., J. M. Chobert, Y. Popineau, M. G. Nicolas, and T. Haertlé. 2001. Improvement of functional properties of  $\beta$ -lactoglobulin glycated through the Maillard reaction is related to the nature of the sugar. *Int. Dairy J.* 11:145–152. [https://doi.org/10.1016/S0958-6946\(01\)00040-1](https://doi.org/10.1016/S0958-6946(01)00040-1).
- Corzo-Martínez, M., F. J. Moreno, M. Villamiel, and F. M. Harte. 2010. Characterization and improvement of rheological properties of sodium caseinate glycated with galactose, lactose and dextran. *Food Hydrocoll.* 24:88–97. <https://doi.org/10.1016/j.foodhyd.2009.08.008>.
- Da Silva Pinto, M., S. Bouhallab, A. F. de Carvalho, G. Henry, J.-L. Putaux, and J. Leonil. 2012. Correction to glucose slows down the heat-induced aggregation of  $\beta$ -lactoglobulin at neutral pH. *J. Agric. Food Chem.* 60:1335. <https://doi.org/10.1021/jf300298q>.
- Farías, M. E. 2012. Autoensamblaje del caseinmacropeptido (CMP) y su impacto en la gelificación y espumado. PhD dissertation. Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires.
- Fechner, A., A. Knoth, I. Scherze, and G. Muschiolik. 2007. Stability and release properties of double-emulsions stabilized by caseinate-dextran conjugates. *Food Hydrocoll.* 21:943–952. <https://doi.org/10.1016/j.foodhyd.2006.10.021>.
- French, S. J., W. J. Harper, N. M. Kleinholz, R. B. Jones, and K. B. Green-Church. 2002. Maillard reaction induced lactose attachment to bovine  $\beta$ -lactoglobulin: Electrospray ionization and matrix-assisted laser desorption/ionization examination. *J. Agric. Food Chem.* 50:820–823. <https://doi.org/10.1021/jf010571q>.
- Ganzevles, R. A., M. A. Cohen Stuart, T. van Vliet, and H. H. J. de Jongh. 2006. Use of polysaccharides to control protein adsorption to the air-water interface. *Food Hydrocoll.* 20:872–878. <https://doi.org/10.1016/j.foodhyd.2005.08.009>.
- Hiller, B., and P. C. Lorenzen. 2010. Functional properties of milk proteins as affected by Maillard reaction induced oligomerization. *Food Res. Int.* 43:1155–1166. <https://doi.org/10.1016/j.foodres.2010.02.006>.
- Jauregui-Rincón, J., E. Salinas-Mirallas, N. Chávez-Vela, and M. Jiménez-Vargas. 2019. Glycomacropeptide: Biological activities and uses. Pages 1–21 in *Whey—Biological Properties and Alternative Uses*. Springer.
- Jensen, H. B., K. S. Pedersen, L. B. Johansen, N. A. Poulsen, M. Bakman, D. E. W. Chatterton, and L. B. Larsen. 2015. Genetic variation and posttranslational modification of bovine  $\kappa$ -casein: Effects on casein-macropeptide release during renneting. *J. Dairy Sci.* 98:747–758. <https://doi.org/10.3168/jds.2014-8678>.
- Katayama, S., J. Shima, and H. Saeki. 2002. Solubility improvement of shellfish muscle proteins by reaction with glucose and its soluble state in low-ionic-strength medium. *J. Agric. Food Chem.* 50:4327–4332. <https://doi.org/10.1021/jf0111717o>.
- Kreuz, M., T. Strixner, and U. Kulozik. 2009. The effect of glycosylation on the interfacial properties of bovine caseinmacropeptide. *Food Hydrocoll.* 23:1818–1826. <https://doi.org/10.1016/j.foodhyd.2009.01.011>.
- Labuza, T. P. 1970. Properties of water as related to the keeping quality of foods. Pages 618–635 in *Proceedings of the Third International Congress of Food Science & Technology*, Washington, DC.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685. <https://doi.org/10.1038/227680a0>.
- Liu, G., and Q. Zhong. 2015. High temperature-short time glycation to improve heat stability of whey protein and reduce color formation. *Food Hydrocoll.* 44:453–460. <https://doi.org/10.1016/j.foodhyd.2014.10.006>.
- Martínez-Alvarenga, M. S., E. Y. Martínez-Rodríguez, L. E. García-Amezquita, G. I. Olivas, P. B. Zamudio-Flores, C. H. Acosta-Muniz, and D. R. Sepulveda. 2014. Effect of Maillard reaction conditions on the degree of glycation and functional properties of whey protein isolate—Maltodextrin conjugates. *Food Hydrocoll.* 38:110–118. <https://doi.org/10.1016/j.foodhyd.2013.11.006>.
- Medrano, A., C. Abirached, L. Panizzolo, P. Moyna, and M. C. Añón. 2009. The effect of glycation on foam and structural properties of  $\beta$ -lactoglobulin. *Food Chem.* 113:127–133. <https://doi.org/10.1016/j.foodchem.2008.07.036>.
- Moreno, F. J., R. López-Fandiño, and A. Olano. 2002. Characterization and functional properties of lactosyl caseinmacropeptide conjugates. *J. Agric. Food Chem.* 50:5179–5184. <https://doi.org/10.1021/jf020118u>.
- Neelima, R., Y. S. Sharma, Rajput, and B. Mann. 2013. Chemical and functional properties of glycomacropeptide (GMP) and its role in the detection of cheese whey adulteration in milk: A review. *Dairy Sci. Technol.* 93:21–43. <https://doi.org/10.1007/s13594-012-0095-0>.
- Nooshkam, M., A. Babazadeh, and H. Jooyandeh. 2018. Lactulose: Properties, techno-functional food applications, and food grade delivery system. *Trends Food Sci. Technol.* 80:23–34. <https://doi.org/10.1016/j.tifs.2018.07.028>.
- Nooshkam, M., M. Varidi, and D. K. Verma. 2020. Functional and biological properties of Maillard conjugates and their potential ap-

- plication in medical and food: A review. *Food Res. Int.* 131:109003. <https://doi.org/10.1016/j.foodres.2020.109003>.
- Roos, Y. H., J. W. Finley, and J. M. deMan. 2018. Water. Pages 1–37 in *Principles of Food Chemistry*, Food Science Text Series. [https://doi.org/10.1007/978-3-319-63607-8\\_1](https://doi.org/10.1007/978-3-319-63607-8_1).
- Sato, R., T. Sawabe, H. Kishimura, K. Hayashi, and H. Saeki. 2000. Preparation of neoglycoprotein from carp myofibrillar protein and alginate oligosaccharide: Improved solubility in low ionic strength medium. *J. Agric. Food Chem.* 48:17–21. <https://doi.org/10.1021/jf990081m>.
- Savre, M. 2016. Maillard-Induced Glycation of Whey Protein Using Maltodextrin and Effect on Solubility, Thermal Stability, and Emulsification Properties. MS thesis. University of Minnesota.
- Sikand, V., P. S. Tong, S. Roy, L. E. Rodriguez-Saona, and B. A. Murray. 2011. Solubility of commercial milk protein concentrates and milk protein isolates. *J. Dairy Sci.* 94:6194–6202. <https://doi.org/10.3168/jds.2011-4477>.
- Tanabe, M., and H. Saeki. 2001. Effect of Maillard reaction with glucose and ribose on solubility at low ionic strength and filament-forming ability of fish myosin. *J. Agric. Food Chem.* 49:3403–3407. <https://doi.org/10.1021/jf001202h>.
- Thomä-Worringer, C., J. Sørensen, and R. López-Fandiño. 2006. Health effects and technological features of caseinomacropeptide. *Int. Dairy J.* 16:1324–1333. <https://doi.org/10.1016/j.idairyj.2006.06.012>.
- Vreeman, H. J., P. Both, J. A. Brinkhuis, and C. van der Spek. 1977. Purification and some physicochemical properties of bovine  $\kappa$ -casein. *Biochim. Biophys. Acta Protein Struct.* 491:93–103. [https://doi.org/10.1016/0005-2795\(77\)90044-7](https://doi.org/10.1016/0005-2795(77)90044-7).
- Zavala Pope, J. M. 2009. Cambios organolépticos y nutricionales producidos por los tratamientos térmicos durante el procesamiento de la leche. Sitio Argentino de Producción Animal. [www.produccion-animal.com.ar/produccion\\_bovina\\_de\\_leche/leche\\_subproductos/00-leche.htm](http://www.produccion-animal.com.ar/produccion_bovina_de_leche/leche_subproductos/00-leche.htm).

## ORCIDIS

- Karen Keel  <https://orcid.org/0000-0003-0802-0332>
- Federico M. Harte  <https://orcid.org/0000-0001-6822-0083>
- Julio Berbejillo  <https://orcid.org/0000-0002-6487-8811>
- Tomás López-Pedemonte  <https://orcid.org/0000-0002-1512-7536>