Influence of desialylation of caseinomacropeptide on the denaturation and aggregation of whey proteins

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ABSTRACT

The effect of the addition of caseinomacropeptide (CMP) or desialylated-CMP on the heat-induced denaturation and aggregation of whey proteins was investigated in the pH range 3 to 7 after heating at 80°C for 30 min. The rate and temperature of denaturation, the extent of aggregation and the changes in secondary structure of the whey proteins heated in presence of CMP or desialylated-CMP were measured. The sialic acid bound to CMP favored the denaturation and aggregation of the whey proteins when the whey proteins were oppositely charged to CMP at pH 4. A transition occurred at pH 6, below which the removal of sialic acid enhanced the stabilizing properties of the CMP against the denaturation and aggregation of the whey proteins. At pH > 6, the interactions between desialylated-CMP and the whey proteins led to more extensive denaturation and aggregation. Sialic acid bound to CMP influenced the denaturation and aggregation behavior of whey proteins in a pH-dependent manner and this should be considered in future studies on the heat stability of such systems containing CMP.

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INTRODUCTION

Bovine whey proteins are known for their nutritional and bioactive properties, which make them ideal ingredients for nutritional beverages such as infant milk formula and protein drinks for athletes and the elderly. However, these ingredients need to be able to withstand thermal treatments such as pasteurization and ultra-high heat treatment applied for reasons of microbiological safety control. Whey proteins are thermolabile and form soluble aggregates, undesirable large visible gel particles or continuous gel networks depending on the conditions of heat treatment.

In contrast to this, bovine caseinomacropeptide (**CMP**), commonly referred as glycomacropeptide (**GMP**) when glycosylated, is a 64 amino acid peptide resulting from the enzymatic cleavage of κ-casein into two peptides (CMP and *para*-κ-casein) and is very heat stable. Glycosylated proteins, such as CMP, present bioactive properties specific to their carbohydrate side chains, sometimes also referred to as prosthetic groups (Nagel et al., 1992). N-acetyl Neuraminic acid (**NeuAc**) is the most abundant member of the sialic acid family in mammalians and is responsible for many bioactive properties of bovine CMP, for example, the promotion of gut microbial growth, the improvement of learning abilities and the modulation of the immune system response (Brody, 2000, Thomä-Worringer et al., 2006, O'Riordan et al., 2014). Amongst other functions, sialic acids play roles in stabilization of cells and proteins, and participate to the transport of positively-charged ions (Traving and Schauer, 1999, Cases et al., 2003, Varki, 2008).

CMP represents up to 25% (w/w) of the total protein in cheese whey (Thomä-Worringer et al., 2006). In this fraction, around 50% of CMP is glycosylated, with the peptide containing

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48 up to six glycosylation and three phosphorylation sites in its C terminal part (Figure 1 A). In
49 mature cow milk, NeuAc is generally located at the end of a glycosylation chain, which apart
50 from NeuAc, contains galactose and N-acetyl galactosamine, organized from monosaccharide
51 to tetrasaccharide (Saito and Itoh, 1992). Thanks to its carboxylic functional group (Figure 1
52 B), NeuAc exhibits a pKa of 2.6 and lowers the overall isoelectric point (**pI**) of the
53 glycoproteins. The estimated pI of κ-casein based on the primary sequence is 5.93;
54 phosphorylation lowers the pI to 5.6, while the glycosylation lowers the pI of κ-casein down
55 to 3.5 (Huppertz, 2013). The pI of glycosylated and non-glycosylated CMP were reported to
56 be 3.2 and 4.2, respectively (Kreuß et al., 2009). The degree of phosphorylation and
57 glycosylation of CMP varies widely, and is illustrated by multiple peaks in the elution profile
58 of reversed-phase HPLC (Thomä et al., 2006) and LC-MS (Sunds et al., 2019), as well as by
59 separation of CMP spots by 2-dimensional electrophoretic analysis (Le et al., 2016). The
60 negative charges carried by the charged amino acid residues, the post-translational
61 modifications at neutral pH and the disordered structure of the peptide, all result in a very
62 hydrophilic and heat stable polypeptide. However, NeuAc is sensitive to acid and heat
63 treatment (Siegert et al., 2012, Kilic-Akyilmaz and Karimidastjerd, 2018). Therefore not all
64 CMP contains the same amount of NeuAc due to heat-induced losses (Taylor and Woonton,
65 2009).

66 These properties can improve the solubility of other proteins when heated in the presence of
67 CMP. The post-translational modifications of the heat-stable proteins α_s-, β- and κ-casein are
68 thought to be involved in the control of the aggregation of whey proteins during heat
69 treatment, by limiting the size of the aggregates or the extent of aggregation (Morgan et al.,
70 2005, Guyomarc'h et al., 2009, Koudelka et al., 2009, Kehoe and Foegeding, 2014). In
71 addition, Doi et al. (1981) showed a correlation between degree of glycosylation of κ-casein

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and improved heat stability of β-lg. Croguennec et al. (2014) studied the effect of CMP on the denaturation and aggregation of whey proteins and demonstrated that CMP increased the rate of denaturation of β-lactoglobulin (**β-lg**) *via* hydrophobic and electrostatic interactions at pH 4.0 and 6.7. However, it could limit the aggregation of β-lg at pH 6.7 due to the negative charges carried by CMP around the neutral pH. Therefore, it is possible that the glycosylation of CMP is involved in the control of aggregation of whey proteins.

To the author's knowledge, the effect of the sialic acid content of CMP on the denaturation and aggregation of a mixture of β-lg and α-lactalbumin (**α-la**), such as in whey protein isolate (**WPI**), has not been the subject of any studies yet. The aim of this study is to bring new insights to the effect of the negatively-charged NeuAc on the denaturation and aggregation behavior of β-lg and α-la in WPI in a wide pH range (3 to 7) during heat treatment (80°C for 2-30 min) with a view to developing strategies for enhancement of the heat stability of whey protein systems.

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MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise. Denatured whey proteins were removed from native whey proteins by isoelectric precipitation. Briefly, a solution of 10 (w /v) % WPI (Davisco Bipro, Eden Prairie, MN, USA) was rehydrated in Milli-Q water, heated at 40°C for 2 h and stirred overnight at 4°C. The pH of the solution was adjusted to 4.6 and centrifuged at 4,000×*g* for 40 min to separate aggregated material from soluble whey proteins. The supernatant, containing the native whey proteins, was adjusted to pH 7.0 and dialyzed against 10 mM sodium phosphate (pH 7.0) for 24 h with 2 changes of buffer, then for 24 h in distilled water with two changes of water. The dialyzed solutions were freeze-dried. The protein content was measured using reversed-phase high-performance liquid chromatography (**RP-HPLC**) using a modification of the method of Beyer and Kessler (1989).

A solution of CMP (Lacprodan cGMP-20, Arla Foods Ingredients, Viby J, Denmark) was enzymatically desialylated following the method described initially by Villumsen et al. (2015) and modified by Sunds et al. (2019). Briefly, the sialidase was added in a ratio of 1:57,000 (w/w) to the protein solution rehydrated at 7% at pH 5.8. The sample was incubated at 37°C overnight and freeze-dried.

The desialylation of CMP resulted in a shift in its pI from 3.0 for the untreated CMP to around 3.7 for the desialylated CMP (**d-CMP**) after analysis by 2-dimensional SDS-PAGE as analyzed and reported elsewhere on the same batch of powder (Sunds et al., 2019). The chromatogram presented in Figure 2 A shows the effect of desialylation on the chromatogram

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of CMP and d-CMP. The CMP and the d-CMP powders were rehydrated, dialyzed and freeze-dried following the same process as for the WPI powders.

Reconstitution

Mixtures of WPI with CMP or d-CMP were rehydrated in Milli-Q water. The concentration of whey protein in the mixtures was 0.5% (w/v). However, the protein content of the freeze-dried CMP and d-CMP powders could not be accurately estimated by Kjeldahl due to the unknown nitrogen to protein conversion factor of CMP and d-CMP used in this study, which varies from 6.71 to 7.37 depending on the variant and as a function of the degree of glycosylation (Karman and Van Boekel, 1986). Attempt to quantify CMP and d-CMP by RP-HPLC by measuring the sum of the peak areas at 214 nm shows that both powders contained the peptide in comparable amounts. Therefore, the CMP or d-CMP powders were added to the whey protein sample in a concentration of 0.5% (weight of CMP or d-CMP powder /v). As control samples, solutions of 0.5 and 1% (w/v) whey protein were rehydrated in Milli-Q water.

Higher proteins concentrations were required for the differential scanning calorimetric (**DSC**) and the Fourier transform infrared spectroscopy (**FTIR**) measurements. Hence, solutions of 2.5% (w/v) whey proteins were also prepared, with CMP or d-CMP (2.5%, w/v). As control samples, solutions of 2.5 and 5% (w/v) whey proteins were rehydrated in Milli-Q water.

Heating of protein solutions

For the measurement of the degree of denaturation of the whey proteins, the ξ-potential, the molecular weight distribution, the turbidity measurement and the microscopy images, the pH of the 5 mL-solutions was adjusted to 3, 4, 5, 6 and 7 and subsequently heated in a water bath

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129 at 80°C and aliquots of 0.7 mL were removed at 2, 5, 10, 15, 20 and 30 min for analysis. The
130 aliquots were immediately cooled to room temperature.

131 For the FTIR measurement, the pH of the samples was adjusted to 4, 5, 6 or 7 and a volume
132 of 200 µl was heated for 30 min at 80°C in a water bath. The samples were heated for only 5
133 min at pH 5 to avoid the gelling of the samples. The samples were immediately cooled to
134 room temperature. For the DSC measurement, the pH of the samples was adjusted to 4, 5, 6
135 or 7 and heated in the equipment as described below.

136 ***Degree of Denaturation***

137 The residual content of native whey proteins after heating was measured by RP-HPLC. The
138 samples were diluted in a sodium acetate/acetic acid buffer at pH 4.6 with a ratio 1:1 to
139 precipitate all denatured and subsequently aggregated proteins (Tolkach et al., 2005, Kehoe et
140 al., 2011). The samples were centrifuged at 14,000×*g* for 30 min at 20°C and the supernatant
141 was filtered through 0.45 µm hydrophilic filters (PES membrane filter type, Sartorius,
142 Göttingen, Germany). A C5 PolymerX RP1 column from Phenomenex (Torrance, California,
143 USA) was used. Buffer A contained 0.1% (v/v) TFA in water and buffer B contained 90%
144 (v/v) ACN and 0.1% (v/v) TFA. The gradient of buffer B was 20% for 3 min, 20 to 40% in
145 10 min, 40 to 60% in 20 min, 60 to 100% in 2 min, 100% for 5 min, 100 to 20% in 0.5 min.
146 The temperature of the column was maintained at 28°C during the run and the flow rate was
147 1 mL/min. The absorbance was measured at 280 nm and 214 nm. The whey protein standards
148 were β-lg, α-la and CMP. The injection volume was 20 µl. The peaks were integrated and the
149 ratio C_t/C_0 was plotted against the heating time, with C_t the residual amount of native whey
150 proteins at a time point t between 0 and 30 min, and C_0 the initial amount of native whey
151 proteins. The rate of denaturation was estimated to be the slope of the tangent line along the

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first 5 min of heating, during which the amount of native whey proteins decreased the most. The amount of denatured and subsequently aggregated protein after 30 min heating was also reported. A typical chromatogram obtained after mixing whey protein and CMP is shown in Figure 2 B.

Differential Scanning Calorimetry

For the DSC measurements, 20-30 mg of sample were placed into an aluminium pan and heated in parallel to an empty reference pan. Despite the starting concentration of the samples (2.5%, w/v) being relatively low, the denaturation peak of β-lg could still be identified, while the denaturation peak for α-la could not be identified in this study. The DSC used for this experiment was a DSC Q2000 (TA Instrument, Newcastle, Delaware, USA) equipped with a refrigerator and a computer. The thermograms were analyzed by the software TA Universal Analysis (TA Instrument, New Castle, DE, USA). The temperature of denaturation of β-lg at pH 3 was not tested as measurements using RP-HPLC showed that there was no loss of native β-lg and formation of aggregates after heating for 30 min at 80°C.

Attenuated Total Reflection – Fourier Transform Infrared Spectroscopy

Measurements of the FTIR were carried before and after heating using a Bruker Tensor 27 instrument (Billerica, MA, USA) equipped with a thermally controlled attenuated total reflection cell BioATRcell II (Harrick Scientific, New York, NYS, USA). An average of 120 scans by samples was taken. The spectra were analyzed using the OPUS 7.5 software (Bruker) after atmosphere compensation, vector normalization and substraction to non-heated samples or samples containing whey proteins only. At pH 3, the whey proteins exhibited very little denaturation and aggregation after heating, therefore this condition was not tested here.

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Turbidity and ξ-potential

The turbidity of the samples was measured in polystyrene micro-cuvettes in a standard UV/vis-spectrophotometer at 20°C. The turbidity was expressed as the optical density at 600 nm. At pH 3, the whey proteins exhibited very little denaturation and aggregation after heating, therefore this condition was not presented here.

The ζ-potential of each sample was measured before and after heating for 30 min. The ζ-potential was determined using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The measurements were carried out at 20°C after an equilibration time of 120 s at room temperature. The refractive index and the viscosity of the dispersant were assumed the same as that of water, i.e., 1.330 and 1.0031 cP, respectively. The attenuation values were between 5 and 11. After heating at pH 5, all samples exhibited microscopic aggregation during heating and the ζ-potential could not be measured.

Molecular Weight Distribution

The molecular weight distribution of the aggregates was measured by size exclusion chromatography on a HPLC system (Waters Alliance e2695, Milford, MA, USA) equipped with a UV/visible detector (2489, Waters Alliance) and the analysis software Empower (Waters Alliance). Two columns in series, TSKgel G2000SW$_{XL}$ and TSKgel G3000SW$_{XL}$ (Tosoh Bioscience GmbH, Griesheim, Germany) with a guard column were used for the separation and analysis of the proteins. The dimension of the columns was 7.8 x 300 mm each and the exclusion volume was equivalent to 5×10^5 Da. The absorbance was recorded at 280 nm. The buffer was 20 mM sodium phosphate (pH 7.0). The flow rate was 0.5 mL/min and the total duration of each run was 60 min. The coefficient of partition was calculated for

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196 the standards (thyroglobulin, aldolase, BSA, β-lg and α-la) and the whey protein aggregates
197 using the elution volume of blue dextran (2×10^6 Da) as exclusion volume.

198 ***Atomic Force Microscopy***

199 Whey proteins and d-CMP aggregates were imaged by atomic force microscopy (**AFM**)
200 using an Asylum Research MFP-3DAFM (Asylum Research UK Ltd., Oxford, UK) in
201 AC-Mode, as previously described (Kehoe et al., 2011). All samples were diluted to 0.1%
202 protein (w/v) and deposited onto a freshly cleaved mica surface. The samples were
203 subsequently dried in a desiccator. Images were processed using AFM imaging software Igor
204 6.12A (Wavemetrics, Portland, OR) and Argyle light (Asylum Research, Goleta, CA) for 3D
205 images.

206 ***Statistical Analysis***

207 All measurements were done, at least, in three independent replicates. DSC and FTIR
208 measurements were done in two independent replicates due to the higher protein
209 concentration required for these experiments and the limited amount of sample available after
210 the enzymatic treatment. The distribution of the rates of denaturation and the turbidity were
211 presented as medians, with quartiles and whiskers representing, respectively, the 25^{th} and 75^{th}
212 mark and the minimum and maximum values. Percentage of denatured proteins, ξ-potential
213 and molecular weight distribution and peak temperature of denaturation were presented as the
214 mean ±SD.

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RESULTS AND DISCUSSION

Denaturation of β-lg and α-la in the Presence of CMP and d-CMP

At temperature greater than 60°C, whey proteins are known to unfold, exposing their hydrophobic sites and making the thiol groups accessible for new intra- and intermolecular interactions. This results in the irreversible aggregation of the whey proteins, which is strongly dependent on the heating conditions. In their native form, β-lg and α-la are soluble at all pH values, including at their pI, 5.1 and 4.2-4.5 respectively (Eigel et al., 1984). The denaturation and aggregation of whey proteins causes their precipitation at pH 4.6 (Okuda and Zoller, 1921). This allowed the measurement of the native proteins during heating, by precipitation of the denatured and subsequently aggregated proteins at pH 4.6, and thus, the estimation of a rate of denaturation in the very early stage of heating. The rate of denaturation of β-lg and α-la, i.e. the estimated rates of denaturation in the first 5 min of heating, are presented in Figure 3. The amounts of denatured α-la and β-lg after 30 min heating are presented in Figure 4. The temperature of denaturation, at which half of the β-lg in the samples has lost their native conformation, was measured by DSC and presented in Table 1.

As expected, due to its unordered, flexible and highly stable structure (Smith et al., 2002), CMP did not exhibit any denaturation at any pH tested (results not shown). Both β-lg and α-la had a higher rate of denaturation at pH 5 than at all other pH, with over 88% (w/w) of β-lg and 66% (w/w) of α-la denatured after 30 min heating at pH 5.0, regardless of the protein composition and concentration (Figure 3 and 4). In contrast, at pH 3.0, there was little denaturation of whey proteins observed after 30 min heating (Figure 4). Stable particles of partially unfolded whey proteins from pH 2.5 were previously observed (Harwalkar, 1980).

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239 However, for experimental conditions used in this study, only native proteins could be
240 measured by RP-HPLC as explained above (Kehoe et al., 2011). Therefore, if any changes in
241 conformation happened to the whey proteins during heating at pH 3 in this study, these
242 modifications had to be reversible to be undetectable by RP-HPLC. Moreover, Verheul et al.
243 (1998) found a decrease of the initial reaction rate and an increase in the temperature of
244 denaturation of β-lg when the protein was heated at pH 3, which supports our findings and
245 suggests that the heating temperature in this study may have been below the temperature of
246 denaturation of whey proteins.
247
248 Above pH 4, α-la had generally a lower rate of denaturation and relative amount of denatured
249 material than β-lg (Figure 3 and Figure 4). This is in agreement with previous studies
250 reporting the greater sensitivity to denaturation of β-lg compared to α-la (Law and Leaver,
251 2000). However, at pH 4, and in particular in the presence of CMP or d-CMP, the rate of
252 denaturation (Figure 3) was higher, regardless of the protein composition and concentration
253 of the samples, than that of β-lg. Although the conformation of proteins is more stable around
254 their pI (4.2-4.5 for α-la), non-covalent interactions are promoted, resulting in greater
255 precipitation of α-la. A higher protein content (1%, w/v, whey protein) also favored α-la
256 denaturation, as reported in previous studies (Hillier et al., 1979), which could be another
257 reason for the greater rate of denaturation of α-la in the presence of CMP or d-CMP (Figure
258 3). The presence of CMP or d-CMP resulted in a lower temperature of denaturation (Table 1)
259 and a greater amount of denatured β-lg after 30 min heating at pH 4 than those of 0.5% (w/v)
260 whey protein only (Figure 4). However, CMP had a stronger effect on the rate of denaturation
261 of β-lg at pH 4 compared to d-CMP (Figure 3). At pH 4, β-lg is positively charged, however
262 CMP is strongly negatively charged, whereas d-CMP is close to its pI (3.7), thus the attractive

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263 electrostatic interactions are stronger with CMP than with d-CMP, leading to faster
264 denaturation of β-lg. A greater decrease of the ξ-potential before heating was observed for the
265 mixture containing 0.5% (w/v) whey proteins with CMP, which could be a result of the
266 attractive interactions between the whey proteins and CMP or an average of their respective
267 ξ-potential at pH 4 (Figure 5 A). Previous authors found that CMP and β-lg interacted at pH
268 3.5 to form aggregates from few nanometers to 1 μm in diameter before heating, most likely
269 *via* electrostatic interactions or hydrogen bonding (Martinez et al., 2010). Changes in the
270 secondary structure of the unheated proteins upon addition of CMP or d-CMP are presented
271 in Figure 6. At pH 4, a decrease around 1655 cm^{-1}, indicated a loss of α-helix and disordered
272 structures in the mixture of whey proteins and CMP or d-CMP, compared to the sample
273 containing whey proteins only (Barth, 2007). Bovine β-lg and α-la have 8% and 26% of
274 α-helix, and 47% and 60% of random coils in their native form, respectively (Deeth and
275 Bansal, 2018). CMP was reported to be mainly disordered with little secondary structure and
276 its glycosylation has very little effect on the secondary structure (Smith et al., 2002).
277 Therefore, the change in secondary structure at pH 4 before heating could be attributed to
278 either the whey proteins or the CMP, or both, and provides evidence for interactions between
279 whey proteins and CMP or d-CMP before heating. Our results showing a higher rate of
280 denaturation of the whey proteins in the presence of CMP (Figure 3) and a greater extent of
281 denaturation in presence of CMP or d-CMP (Figure 4) were in agreement with recent studies
282 that highlighted that the temperature for the onset of denaturation and the temperature of
283 denaturation of β-lg decreased with the ratio β-lg to CMP at pH 3.5 (Martinez et al., 2010)
284 and the denaturation of β-lg accelerated in the presence of CMP at pH 4.0 (Croguennec et al.,
285 2014).
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287 At pH 5, β-lg was very close to its native pI of 5.2 and its global surface charge was minimal,
288 which promoted non-covalent aggregation. Under these circumstances, the rate of
289 denaturation and the amount of denatured β-lg were higher than those of α-la (Figure 3 and
290 4). The rates of denaturation of β-lg and α-la were lowered at pH 5 and 6 in the presence of
291 CMP or d-CMP (Figure 3), with the exception of α-la in the presence of d-CMP and at pH 6.
292 These lower rates of denaturation could be caused by the stabilization of the whey proteins by
293 additional electrostatic repulsion provided by CMP or d-CMP. These results are supported by
294 a higher temperature of denaturation of β-lg at pH greater than 4 in the presence of CMP or d-
295 CMP (Table 1). The ξ-potential was closer to zero in 0.5% (w/v) whey protein sample in the
296 presence of d-CMP than in the presence of CMP at pH 5 (Figure 5 A). This could be the
297 average of the surface charges of the peptide and the whey proteins, or the proteins could
298 interact more readily by attractive electrostatic and possibly hydrophobic interactions when
299 the sialic acid NeuAc was removed. At pH 5 and 6, the temperature of denaturation of β-lg in
300 the presence of d-CMP was higher than that in the presence of CMP (Table 1). Haque and
301 Khalifa (1992) found that the hydrophobicity of κ-casein fractions decreased with their
302 content of NeuAc. In addition, the glycosylation of CMP caused steric hindrance,
303 electrostatic repulsion and less hydrophobic interaction, which prevented interaction with oil
304 at the interface of emulsions (Kreuß et al., 2009). Therefore, the highly negatively-charged
305 glycosylation of CMP may prevent hydrophobic interactions by increasing the electrostatic
306 repulsions between CMP and whey proteins. The negative charges carried by the amino acids
307 and the negatively charged phosphorylated residues of d-CMP could also have contributed to
308 the stability of the whey proteins on their own. Koudelka et al. (2009) showed that the
309 phosphorylated residues, the amphipathic nature and the flexibility of the caseins, α_{s1}- and
310 β-casein, are key features of their chaperone activity on proteins.

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311

312 At pH 5, the amount of denatured whey proteins after 30 min of heating in the presence of
313 d-CMP was reduced as compared to that of 0.5% (w/v) whey protein only (Figure 4).
314 However, at pH 6, the stabilization of β-lg by d-CMP was only effective in the initial stage of
315 heating (up to 5 min) and the amount of denatured β-lg increased after 30 min heating with
316 d-CMP (Figure 3 and 4). The conformation of whey proteins is more stable at pH values
317 close to their pI, and less stable at pH greater than 5, due to increased intramolecular
318 repulsion leading to unfolding and increased reactivity of the thiol groups (Hoffmann and van
319 Mil, 1997). This was illustrated by a decrease of the temperatures of denaturation (Table 1)
320 for all samples at pH greater than 4. Thus, although the presence of d-CMP initially stabilized
321 the native conformation of β-lg, the interactions between β-lg and d-CMP were affected by
322 the heat-induced unfolding of the whey proteins and the formation of new intermolecular
323 disulphide bonds, and promoted the denaturation of β-lg on prolonged heating at pH 6.
324 Croguennec et al. (2014) also observed that pH affected the heat-induced interactions
325 between CMP and β-lg with a stabilizing effect on the native conformation of β-lg at pH 4.0
326 and a destabilizing effect at pH 6.7. The mixture of whey proteins and d-CMP had a lower ξ-
327 potential than the sample with 0.5% (w/v) whey protein only at pH 6 (Figure 5 A), which
328 could be due to greater electrostatic interactions or could be the average of the ξ-potential of
329 all proteins in solution. However, interactions between CMP or d-CMP and the whey proteins
330 above pH 5 were evident from a strong decrease in the intramolecular β-sheet signal (Figure
331 6) around 1630 cm^{-1}. This contrasts with the changes in secondary structure obtained at pH 4,
332 where a loss of α-helix or random coil structure was observed (Figure 6), and highlights the
333 effect of pH on the nature of the interaction between the whey proteins and CMP or d-CMP.

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335 At pH 7, CMP, and more particularly d-CMP, promoted the denaturation of β-lg (Figure 3
336 and 4). Thus, from this method based on the precipitation of the aggregates at their pI, there
337 was no evidence for stabilization of the whey proteins, even during the initial stages of
338 heating. In contrast to this, the temperature of denaturation of β-lg, i.e., the temperature
339 measured by DSC at which 50% of β-lg is denatured, was higher in the presence of CMP or
340 d-CMP at pH 7 (Table 1). However, the temperature of denaturation of β-lg heated in the
341 presence of d-CMP was close to that of the control containing 0.5% (w/v) whey protein only.
342 Therefore, the sialic acid hindered the denaturation of β-lg at pH 7, and its removal resulted
343 in lower electrostatic interactions, which could have facilitated the hydrophobic interactions
344 between d-CMP and the unfolded β-lg. Other authors have previously demonstrated the role
345 of hydrophobic and electrostatic interactions on stabilizing the native conformation of β-lg in
346 the presence of peptides from hydrolyzed whey proteins (Barbeau et al., 1996). The higher
347 charge density of the peptides and the hydrophobic interactions of β-lg with the peptides were
348 assumed to induce a more compact form of β-lg. Above pH 6.8, the protective effect of the
349 negatively-charged peptides was lower, which is in agreement with our results. Other authors
350 emphasized that CMP accelerated the denaturation rate of β-lg and promoted the unfolding of
351 β-lg at pH 6.7 due to an increase in negative charges, which destabilizes the native state of
352 β-lg (Croguennec et al., 2014). The authors concluded that CMP interaction is stronger with
353 the unfolded form of β-lg than with the compact native form. In addition, Martinez et al.
354 (2009) reported a decrease in the temperature of denaturation and the onset temperature of
355 denaturation of β-lg measured by DSC in the presence of CMP. However, other authors
356 found that CMP increased the temperature of denaturation when β-lg was heated with other
357 whey proteins (Svanborg et al., 2016). These results could be explained by differences in the
358 composition of the starting materials.

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359

360 The presence of CMP or d-CMP did not affect the early stage of denaturation of α-la at pH
361 greater than 5 to the same extent as it affected the early denaturation of β-lg (Figure 3). The
362 differences in chemical composition between the two whey proteins, in particular the absence
363 of a free thiol group on α-la and the ability of β-lg to bind small hydrophobic molecules,
364 could explain the differences observed (Muresan et al., 2001).

365 ***Aggregation Behavior of Whey Proteins in the Presence of CMP or d-CMP***

366 Figure 7 and 8 present the turbidity of the protein solutions after heating at 80°C for 30 min
367 and Figure 9 illustrates the molecular weight distribution of the samples after heating. The
368 optical density (OD) at 600 nm was a sensitive indicator of the extent of whey protein
369 aggregation (Li et al., 2019).

370

371 As expected, the proteins did not form large aggregates at pH 3 (Figure 9 A). At pH 5, the
372 turbidity increased greatly within 2 min of heating, due to a greater instability of β-lg during
373 heating as electrostatic repulsion was at its minimum around its pI (results not shown); all
374 samples gelled after 30 min of heating at pH 5. At pH 4 and within 5 min of heating, the
375 samples containing CMP developed a higher turbidity than the samples containing only whey
376 proteins (Figure 7 A and 8 A). The greater extent of aggregation at this pH could be the result
377 of attractive electrostatic interactions between the whey proteins and CMP. Most of those
378 aggregates had likely been removed after filtration through 0.45 μm filters, prior to size
379 exclusion chromatography; very few aggregates greater than 500 kDa were present in the
380 filtrate (Figure 9 B), which is in contradiction with the high turbidity developed in the
381 samples containing CMP (Figure 7 A). At pH 4, the aggregates in the samples containing
382 CMP presented a higher turbidity than the ones in the samples heated at pH greater than 5

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383 (Figure 7 A), although the amount of denatured protein was lower (Figure 4). Croguennec et
384 al. (2014) observed the formation of aggregates with diameter greater than 5 μm, and a phase
385 separation in a solution of β-lg and CMP heated at pH 4.0.
386
387 At pH 6, the largest aggregates were formed when 1% (w/v) whey protein was heated on its
388 own (Figure 9 C). This is in agreement with the higher amount of denatured protein (Figure
389 4) and the high OD of the samples after 30 min heating (Fig 7 B). The OD was much lower in
390 the samples containing CMP or d-CMP (Figure 7 B and 8 B). The amount of denatured β-lg
391 in the samples containing CMP or d-CMP at pH 6 was higher than that containing 0.5% (w/v)
392 whey protein only (Figure 4). This confirmed that the effect of CMP and d-CMP on the
393 denaturation of the whey proteins also affected the aggregation behavior of the whey
394 proteins. In spite of the interactions between β-lg and CMP or d-CMP leading to more
395 denatured β-lg after 30 min of heating, its stabilization within the first 5 min of heating could
396 have had a durable effect on the structure of the aggregates. An example of a three-dimension
397 AFM image of the aggregates of whey proteins and d-CMP at pH 6 is presented in Figure 10.
398 The height across section (Figure 10 B) shows that the aggregates are polydisperse, with sizes
399 ranging from 5 to 20 nm. High resolution phase and amplitude image (Figure 10 A and C)
400 show that the aggregates consist of individual monomers of proteins, presumably β-lg.
401
402 At pH 7, the OD and the aggregate size range were lower than at pH 6 for all samples (Figure
403 7 and 9). The OD of the samples containing 0.5% whey protein, with or without CMP, or
404 d-CMP, were very close (Figure 7 C). However, the relative amount of medium size
405 aggregates was lower and the relative amount of monomers, dimers or trimers smaller than
406 60 kDa was higher in 0.5% (w/v) whey protein than in the samples containing d-CMP (Figure

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407 9 D). This is in agreement with more denatured β-lg being measured in the case of the
408 samples containing d-CMP (Figure 4), and can be explained by stronger interactions between
409 the whey proteins in the presence of d-CMP. It was previously reported that, at pH 7.0, the
410 heat-induced gelation of β-lg would only occur in the presence of CMP, while a solution of
411 β-lg on its own would not gel, at least under the experimental conditions of this study
412 (Martinez et al., 2010); the authors highlighted that the temperature required for the gelation
413 of the protein systems was lowered in the presence of CMP. In contrast, Croguennec et al.
414 (2014) found smaller particle size of aggregates and a decrease in turbidity after heating β-lg
415 at pH 6.7 in the presence of CMP, although the corresponding activation energy in the
416 aggregation-limited temperature range (above 80°C) decreased in the presence of CMP. Both
417 of these studies hypothesized that the negative charges of β-lg and CMP around pH 7
418 destabilized the native form of β-lg. The main difference between the studies was the
419 resulting type of protein gel. This could be due to the variation in heating conditions and
420 starting materials. Overall, the differences in molecular weight of the aggregates were minor
421 at pH 7 in the present study.
422
423 After heating, all aggregates formed in solutions at pH greater than 5, with or without CMP,
424 exhibited a more negative zeta potential (Figure 5 B). This is an effect of heating on whey
425 proteins that is well documented (Ryan et al., 2012, Kehoe and Foegeding, 2014). The
426 changes in secondary structure of the proteins after heating give further insight on the effect
427 of CMP or d-CMP on whey protein aggregation as a function of pH (Figure 11). As
428 explained above, the changes in β-sheets are mainly attributed to β-lg and α-la, containing
429 respectively 45% and 14% β-sheets in their native form (Deeth and Bansal, 2018).
430 Intramolecular β-sheets absorb around 1630 cm^{-1}, and the heat-induced formation of

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431 intermolecular β-sheets causes a shift in their absorption to 1620 cm^{-1} (Lefèvre and Subirade,
432 2000, Kehoe et al., 2008). From pH 4 to 6, the presence of CMP or d-CMP prevented the
433 formation of intermolecular β-sheet in heat-induced aggregates. At pH 7, CMP or d-CMP did
434 not prevent the formation of intermolecular β-sheets (Figure 11), which is in agreement with
435 the rate of denaturation and denatured material results (Figure 3 and 4).

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CONCLUSIONS

The desialylation of CMP modified the electrostatic interactions between the peptide and major whey proteins β-lg and α-la during heating as a function of pH. Above the pI of the proteins, the removal of the sialic acid facilitated interactions between CMP and the major whey proteins, particularly β-lg, likely through enhanced hydrophobic interactions. The presence of CMP led to a greater extent of denaturation and aggregation of the whey proteins when they were heated at around neutral pH (i.e., pH favoring their unfolding). In the initial stages of heating and at pH 5-6 (i.e., near their pI), the whey proteins were in a more stable conformation and the interactions with CMP led to an enhanced stability of the whey proteins against denaturation and aggregation. These results contribute to a better understanding of the mechanism of interaction between the major whey proteins and CMP. Advantage should be taken of this knowledge and the innate CMP content of cheese whey to enhance the heat-stability of whey proteins. In particular, any pre-processes resulting in the loss of sialic acid are likely to affect the heat-induced denaturation and aggregation of whey proteins.

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580 **Table 1.** Temperature of denaturation of β-lactoglobulin (β-lg) by differential scanning
581 calorimetry (DSC) for samples containing 2.5-5% (w/v) whey proteins (WP) and a mixture of
582 2.5% (w/v) WP and CMP or desialylated CMP (d-CMP). The samples were heated up to
583 100°C, at pH 4 to 7, and the heating rate was 5°C/min. The temperature of denaturation of β-
584 lg at pH 3 was not tested as previous measurements (Figure 4) showed that β-lg did not
585 denature after heating for 30 min at 80°C. Experimental points were the average of data from
586 two independent trials ±SD.

	Temperature of denaturation of β-lg (°C)			
pH	4	5	6	7
5% WP	87.5 ±0.0	80.3 ±0.2	79.8 ±0.1	77.8 ±0.1
2.5% WP	88.3 ±0.3	80.2 ±0.2	80.9 ±0.1	73.2 ±0.5
2.5% WP + CMP	85.0 ±0.5	82.4 ±0.4	81.3 ±0.0	77.0 ±0.2
2.5% WP + d-CMP	86.3 ±0.8	83.6 ±0.2	82.1 ±0.4	75.1 ±1.0

587

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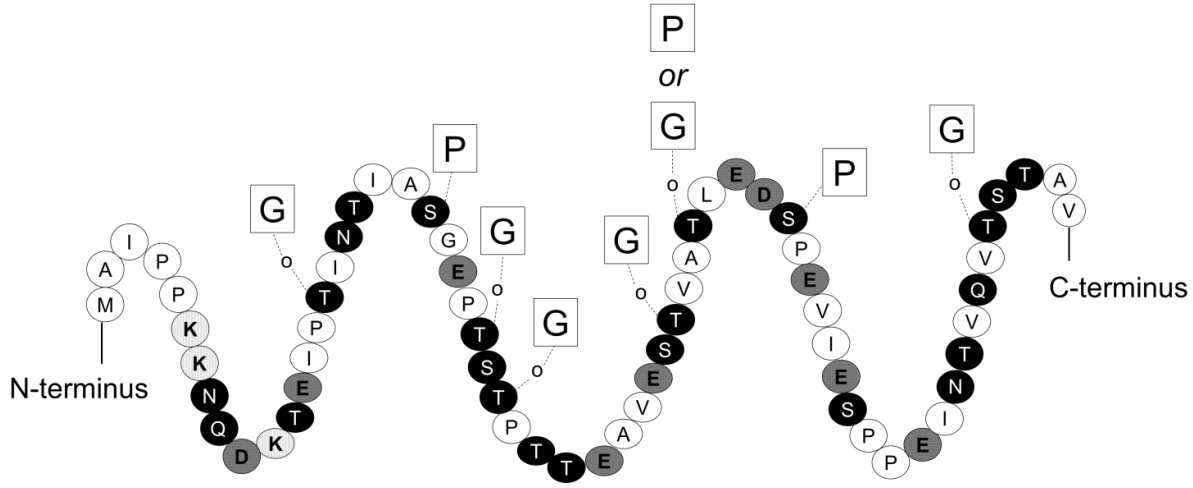

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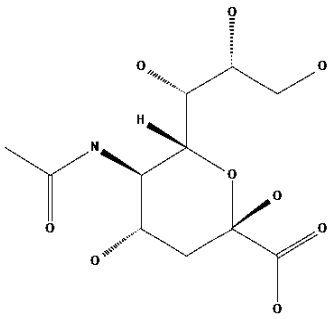

591

592 By Gaspard *et al.*

593 **Figure 1.**

594 (A) Amino acids sequence of caseinomacropeptide (CMP), derived from κ-casein A, with
595 potential sites for post-translational modifications; potential glycosylation site: o G ;
596 potential phosphorylation site: P). The amino acids with (○) non-polar, (●) polar, (◍)
597 negatively-charged and (◍) positively-charged side chains are also indicated on the figure, as
598 reviewed by Holland (2008). (B) 2D structure of N-acetyl-beta-D-Neuraminic acid
599 (PubChem CID: 445063). JDS.2019-17780 Figure 1

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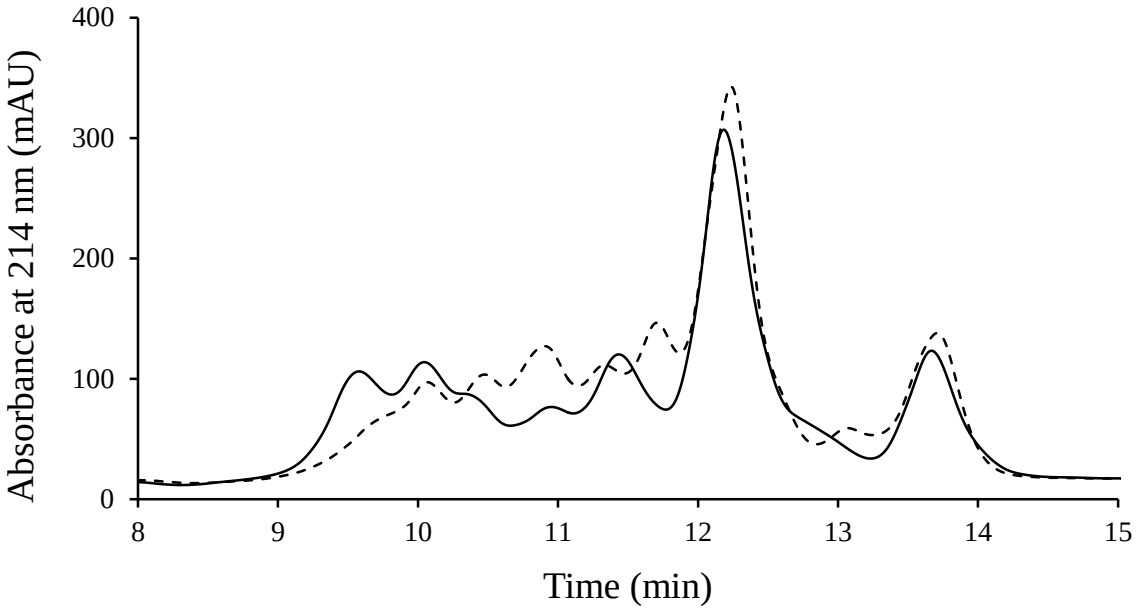

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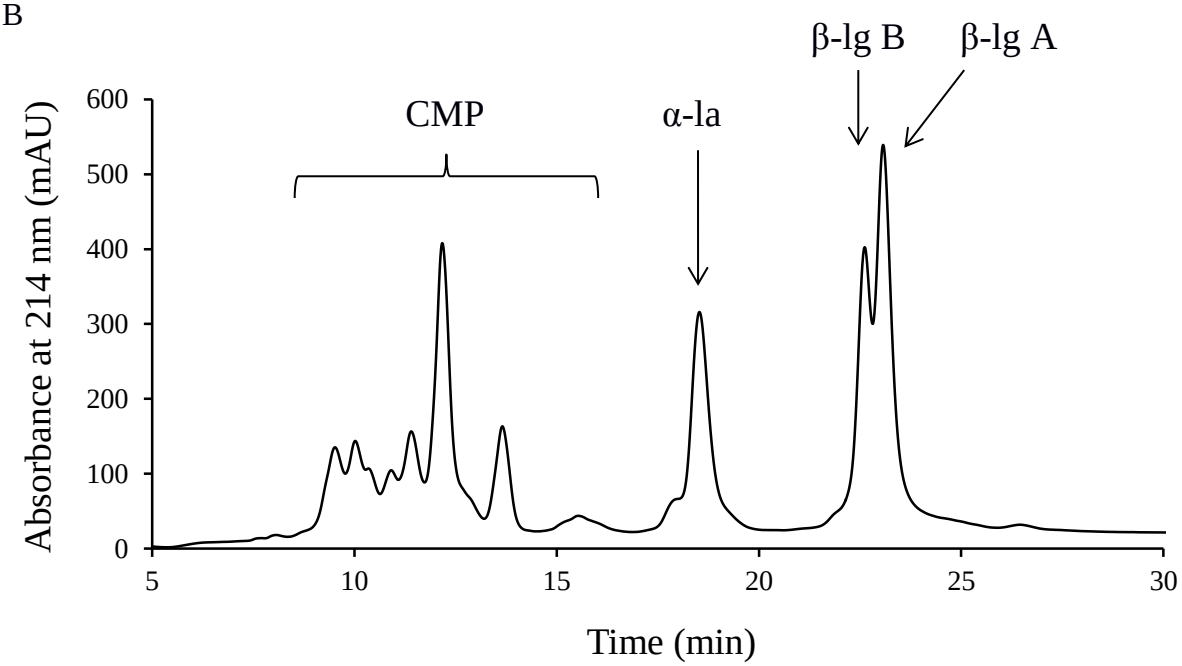

603

604 By Gaspard *et al.*

605 JDS.2019-17780 Figure 2

Revised and accepted in January 2020
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606 **Figure 2.**

607 Chromatogram of (A) solutions of (——) caseinomacropeptide (CMP) and (– –) desialylated
608 CMP and (B) a mixture of CMP and whey proteins, containing α-lactalbumin (α-la) and
609 β-lactoglobulin A and B (β-lg) resolved using reversed-phase high performance liquid
610 chromatography. JDS.2019-17780 Figure 2

611

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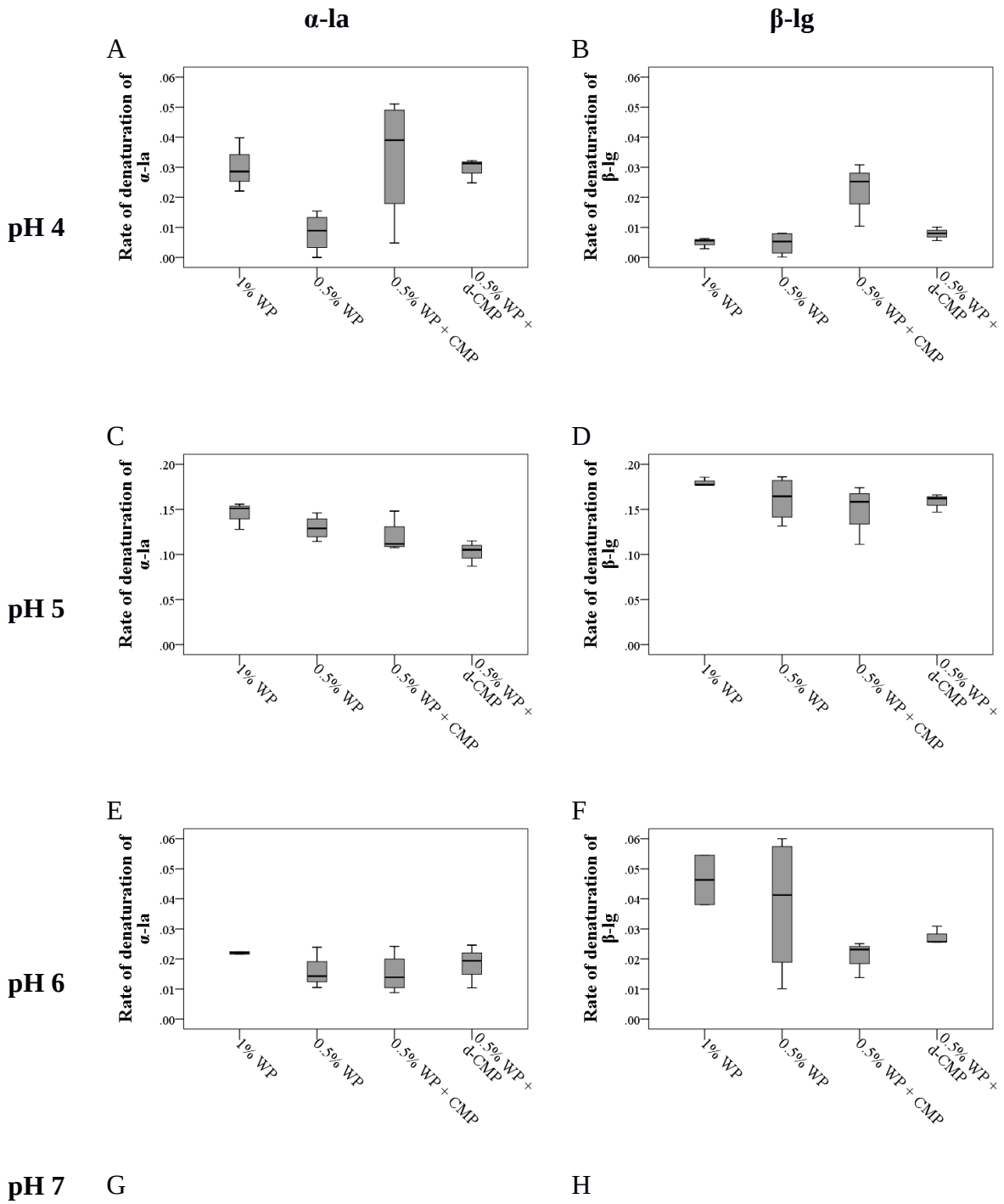

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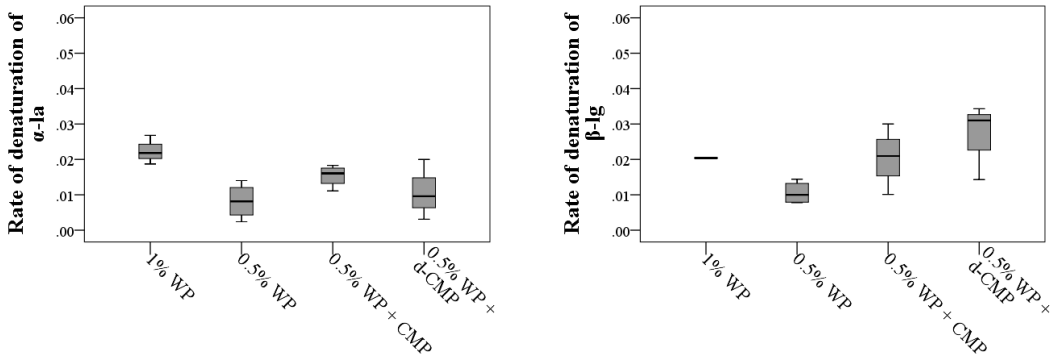

612 JDS.2019-17780 Figure 3

613 By Gaspard *et al.*

614 **Figure 3.**

615 Rates of denaturation (min^{-1}) of (A, C, E, G) α-lactalbumin (α-la) and (B, D, F, H)
616 β-lactoglobulin (β-lg) in the first 5 min of heating at 80°C at (A, B) pH 4, (C, D) pH 5, (E, F)
617 pH 6 and (G, H) pH 7 of 1% (w/v) whey protein (WP) solution, 0.5% (w/v) WP solution, a
618 mixture of 0.5% (w/v) WP and caseinomacropeptide (CMP) and a mixture of 0.5% (w/v) WP
619 and desialylated CMP (d-CMP). Experimental points were the average of data from at least
620 three independent trials. The results were presented as medians, with quartiles and whiskers
621 representing the 25^{th} and 75^{th} mark and the minimum and maximum values, respectively.
622 Figure 4 showed that the whey proteins exhibited very little denaturation and aggregation
623 after heating at pH 3 for 30 min at 80°C, therefore the rates of denaturation of α-la and β-lg at
624 pH 3.0 were not presented here.

625 JDS.2019-17780 Figure 3

626

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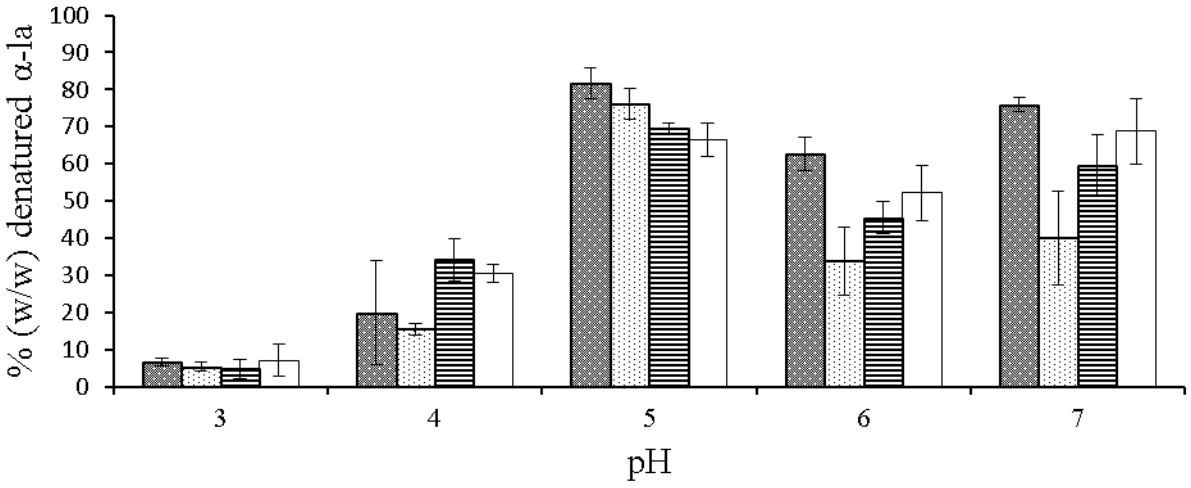

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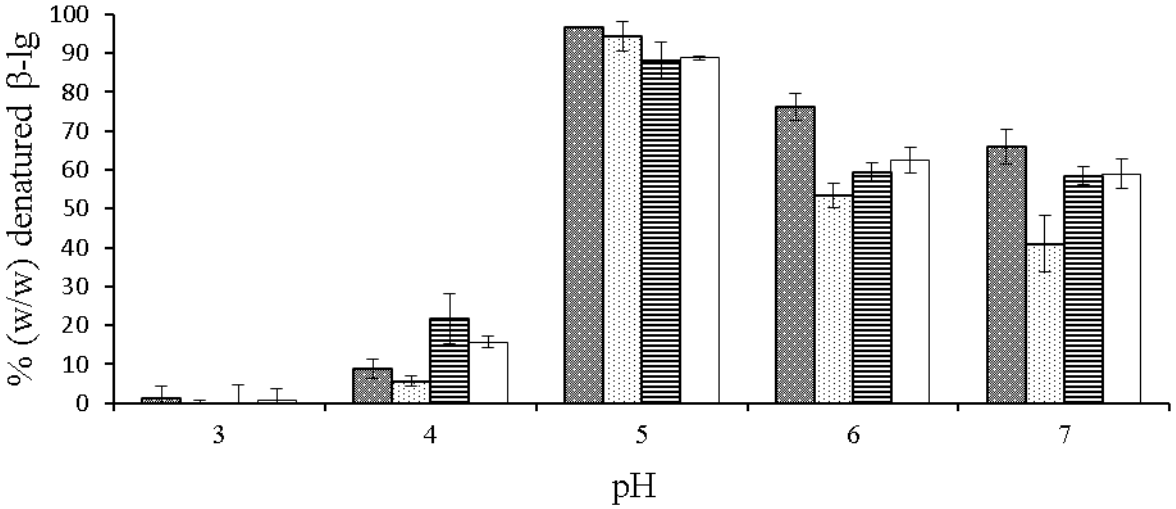

630

631 By Gaspard *et al.*

632 JDS.2019-17780 Figure 4

Revised and accepted in January 2020
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633 **Figure 4.**

634 Percentage of denatured (A) α-lactalbumin (α-la) and (B) β-lactoglobulin (β-lg) after heating
635 (▩) 1% (w/v) whey protein (WP), (▨) 0.5% (w/v) WP, (▤) a mixture of 0.5% (w/v) WP and
636 caseinomacropeptide (CMP) and (□) a mixture of 0.5% (w/v) WP and desialylated CMP
637 (d-CMP) at 80°C for 30 min after adjustment at pH 3, 4, 5, 6 and 7. The annotation w/w
638 refers to weight of denatured protein per total of the corresponding protein. Experimental
639 points were the average of data from at least three independent trials and the error bars
640 correspond to the standard deviations.

641

642 JDS.2019-17780 Figure 4

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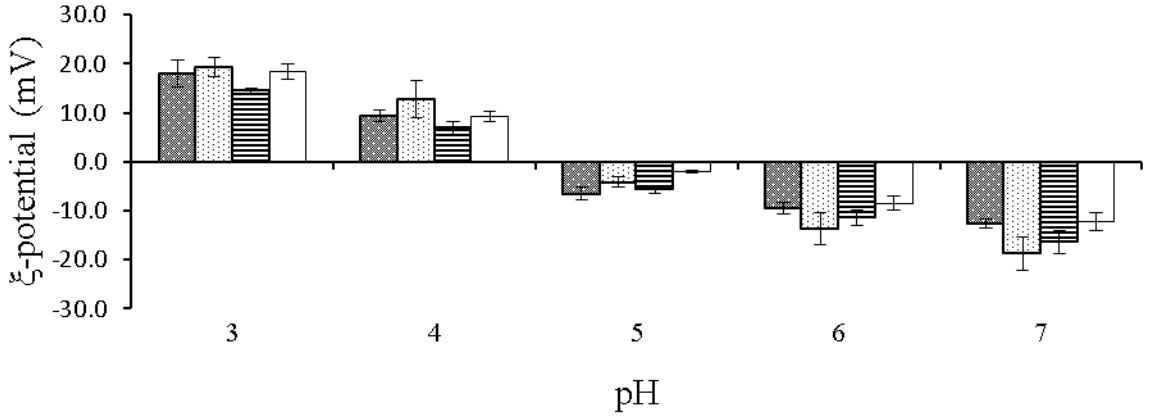

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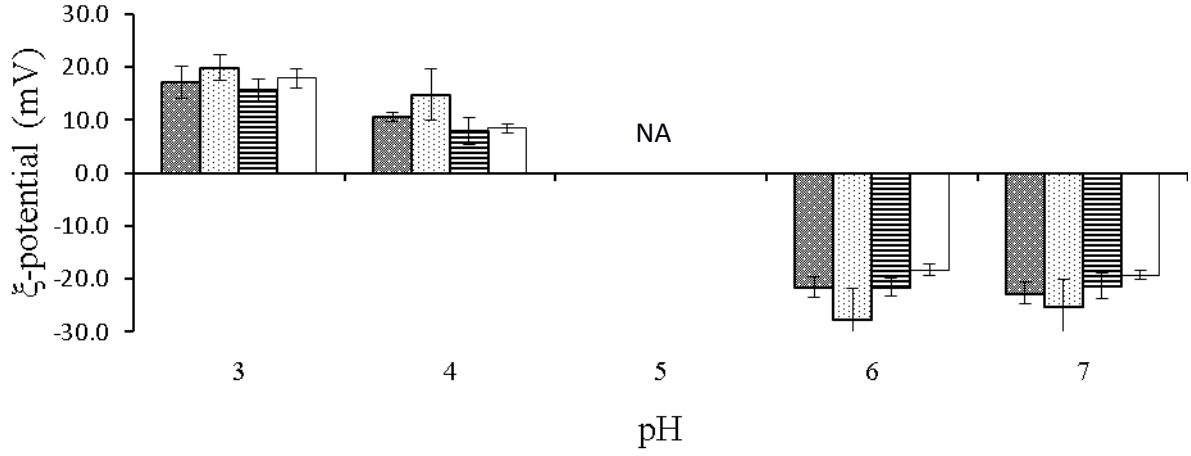

647

648 By Gaspard *et al.*

649 JDS.2019-17780 Figure 5

650

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Figure 5.

ξ-potential of (▩) 1% (w/v) whey protein (WP) solution, (▨) 0.5% (w/v) WP solution, (▤) a mixture of 0.5% (w/v) WP and caseinomacropeptide (CMP) and (□) a mixture of 0.5% (w/v) WP and desialylated CMP (d-CMP) (A) before and (B) after heating at 80°C for 30 min at pH 3, 4, 5, 6 and 7. Experimental points were the average of data from at least three independent trials and the error bars correspond to the standard deviations. NA indicates that the samples exhibited microscopic aggregation during heating and no measurements were taken.

JDS.2019-17780 Figure 5

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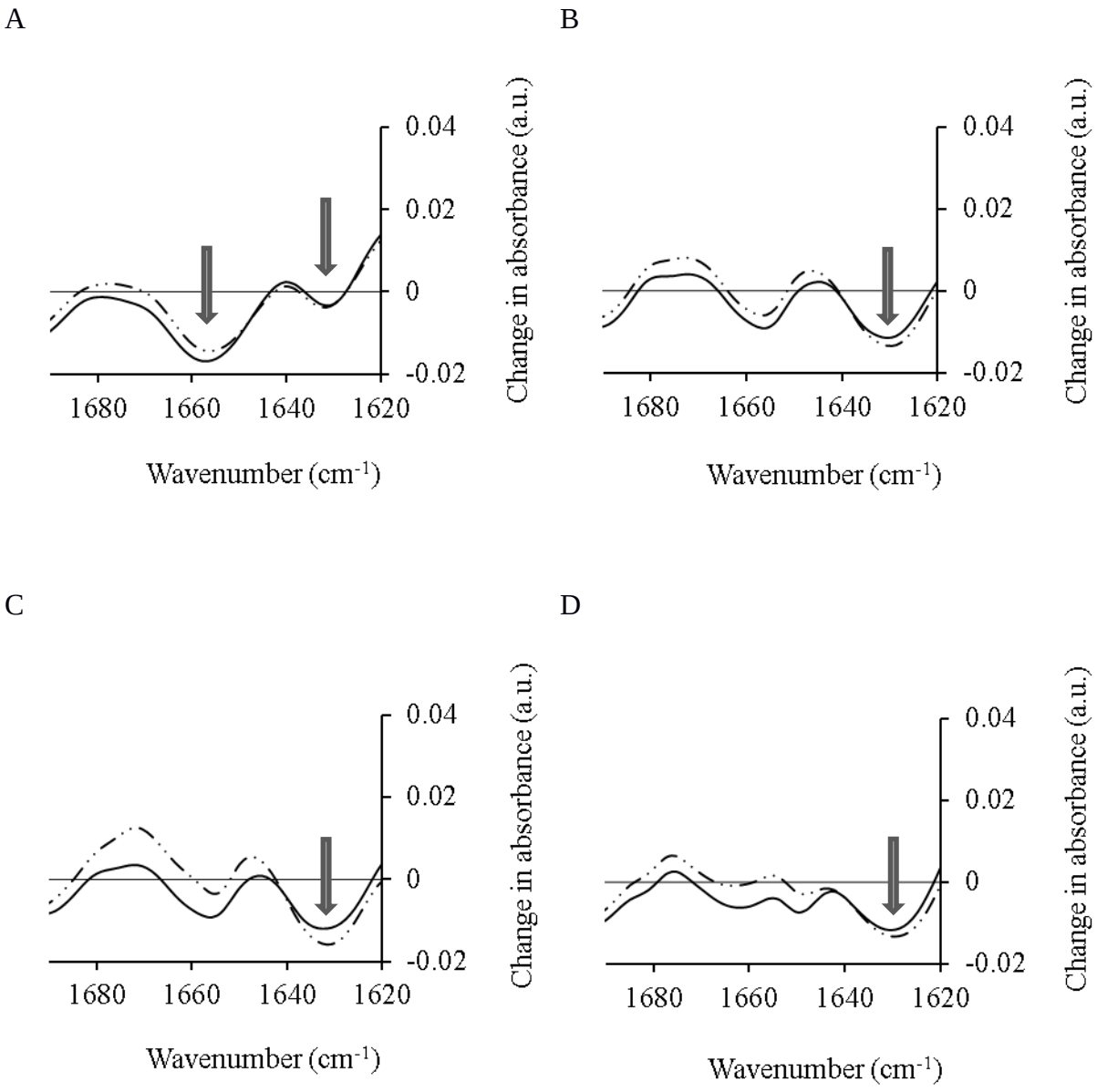

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663 JDS.2019-17780 Figure 6

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Figure 6.

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) spectra of amide I bands of non-heated samples at (A) pH 4, (B) 5, (C) 6 and (D) 7 containing (——) 2.5% (w/v) whey proteins (WP) with caseinomacropeptide (CMP) or (– ·· –) 2.5% (w/v) WP with desialylated CMP (d-CMP), with the spectra of the non-heated solution of 2.5% (w/v) WP substracted. The arrows highlighted the deviations in the region 1655 cm^{-1} and 1630 cm^{-1} upon the addition of the CMP or d-CMP from the whey protein solutions, represented by a straight line through the origin. Experimental points were the average of data from two independent trials. The FTIR measurements were carried out on samples exhibiting some protein denaturation after heating for 30 min at 80°C. At pH 3, the whey proteins exhibited very little denaturation and aggregation after heating, therefore this condition was not tested here (Figure 4).

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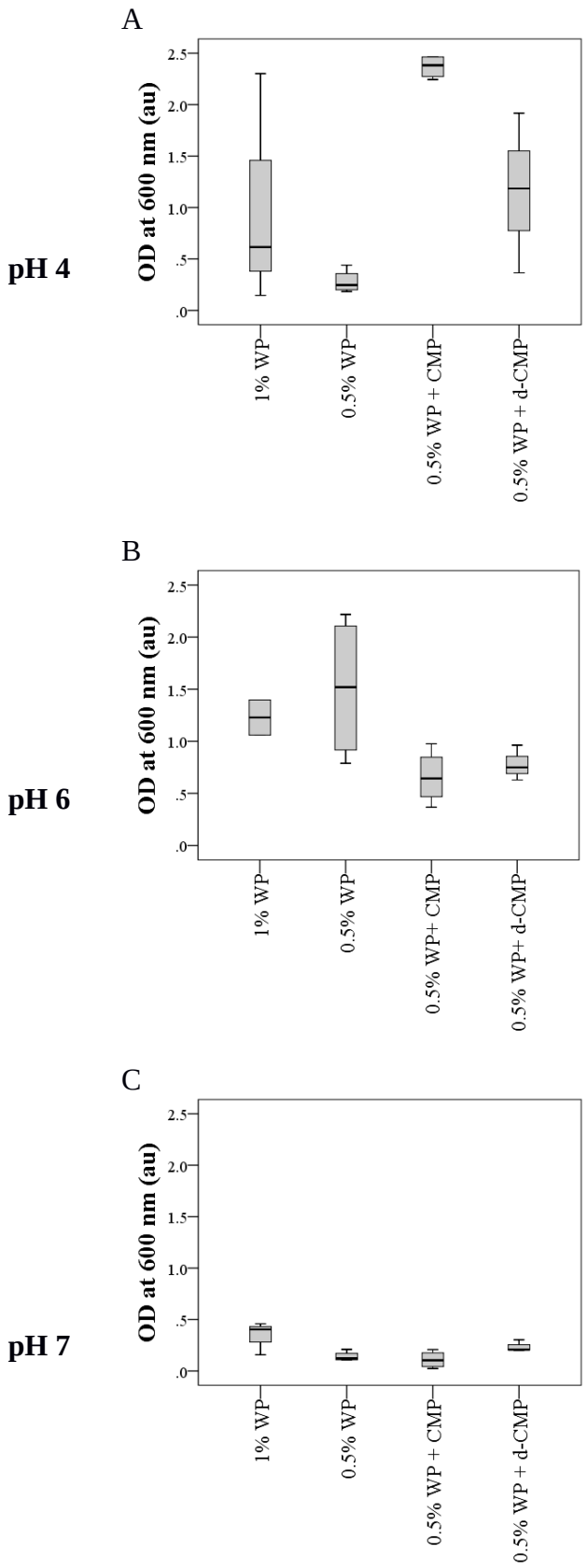

677 JDS.2019-17780 Figure 7

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679 **Figure 7.**

680 Turbidity expressed as optical density (OD) at 600 nm of 1% (w/v) whey proteins (WP)
681 solution, 0.5% (w/v) WP solution, a mixture of 0.5% (w/v) WP and caseinomacropeptide
682 (CMP) and a mixture of 0.5% (w/v) WP and desialylated CMP (d-CMP) after heating at
683 80°C for 30 min at (A) pH 4, (B) 6 and (C) 7. The results were presented as medians, with
684 quartiles and whiskers representing, respectively, the 25^{th} and 75^{th} mark and the minimum
685 and maximum values. Experimental points were the average of data from at least three
686 independent trials. All samples displayed microscopic aggregates at pH 5 after a few minutes
687 of heating, and measurements could not be taken. At pH 3, the whey proteins exhibited very
688 little denaturation and aggregation after heating (Figure 4), therefore the results at this pH
689 condition were not presented here.

690

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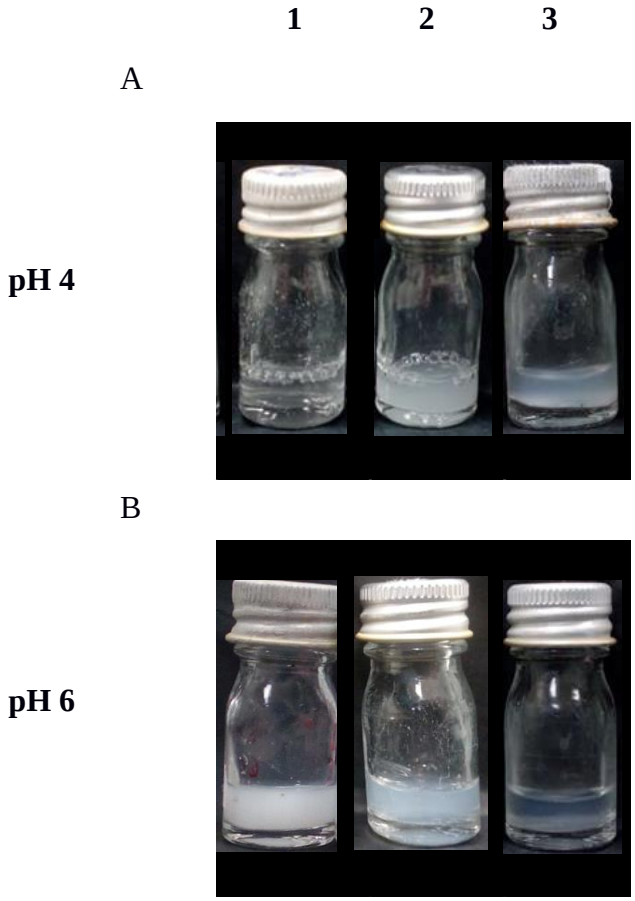
691 JDS.2019-17780 Figure 7



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693 **Figure 8.**

694 Photographs of a solution of (1) 0.5% (w/v) whey protein (WP) solution, (2) a mixture of

695 0.5% (w/v) WP and CMP and (3) a mixture of 0.5% (w/v) WP and desialylated CMP

696 (d-CMP) after heating at 80°C for 30 min at (A) pH 4 and (B) pH 6.

697 JDS.2019-17780 Figure 8

698

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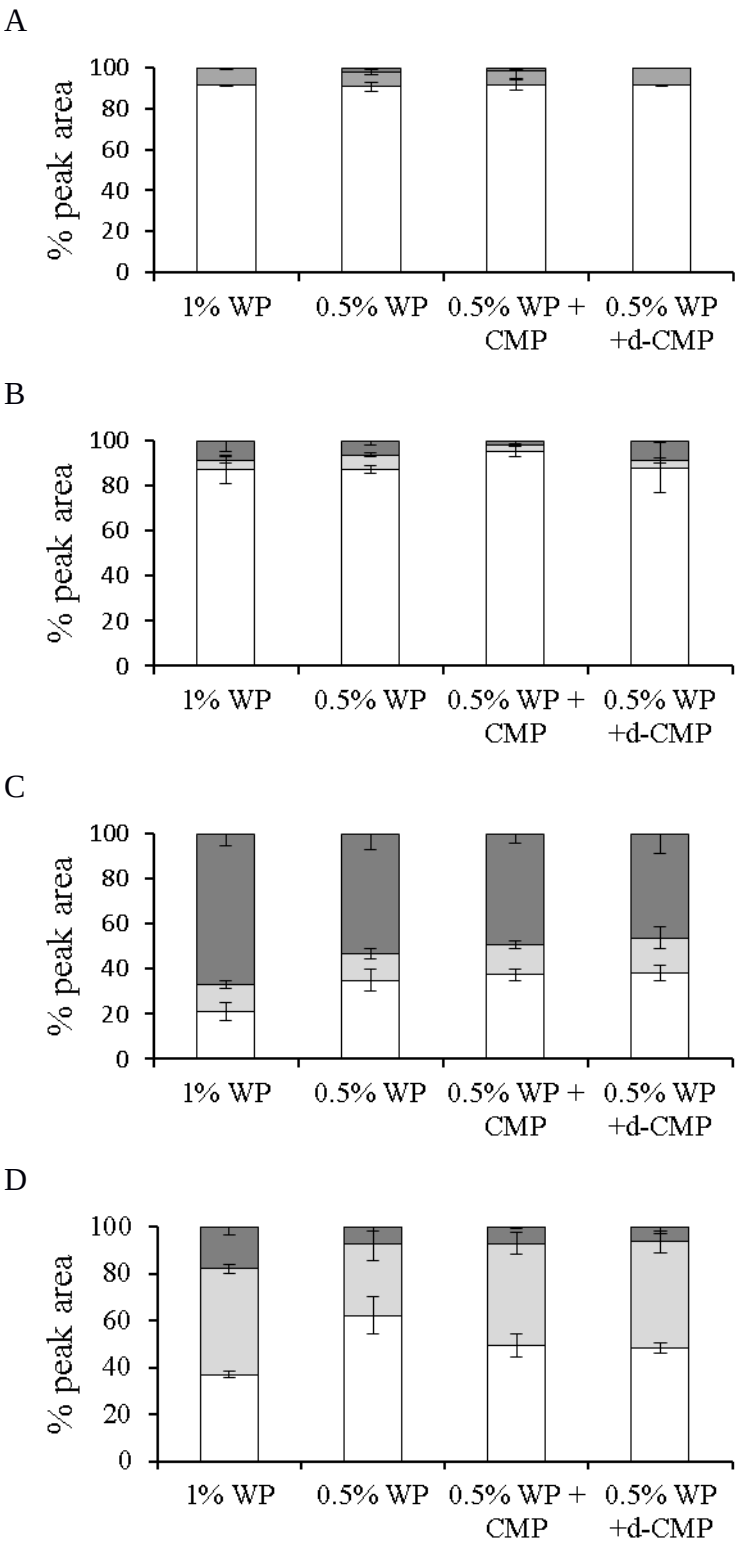

699 By Gaspard *et al.*

700 JDS.2019-17780 Figure 9

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Figure 9.

Molecular weight distribution of soluble whey proteins (WP) by size-exclusion chromatography (SEC-HPLC) in 1% (w/v) WP solution, 0.5% (w/v) WP solution, a mixture of 0.5% (w/v) WP and caseinomacropeptide (CMP) and a mixture of 0.5% (w/v) WP and desialylated CMP (d-CMP) after heating at 80°C for 30 min at (A) pH 3.0, (B) 4.0, (C) 6.0 and (D) 7.0. Distribution represented as: (□) 8 to 60 kDa, (□) 60 to 500 kDa and (□) >500 kDa. Experimental points were the average of data from at least three independent trials and the error bars correspond to the standard deviations. All samples displayed microscopic aggregates at pH 5 after few minutes of heating at 80°C and most of the aggregates formed during heating were filtered out through 0.45 μm, therefore the results were not presented here.

JDS.2019-17780 Figure 9

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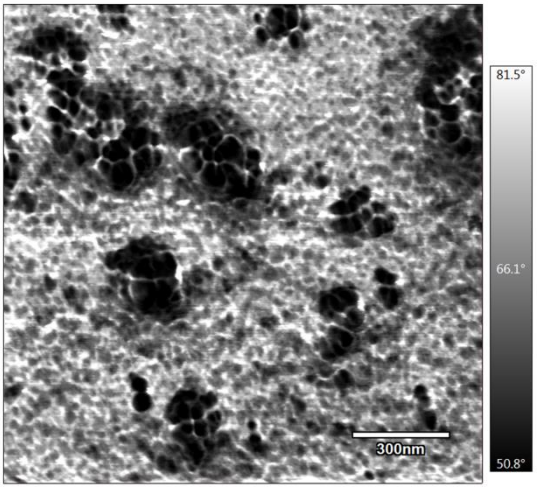

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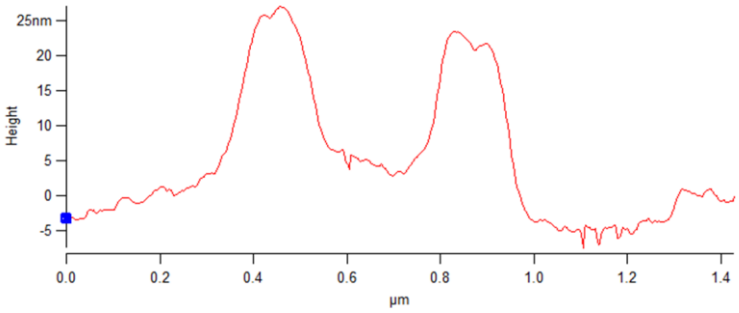

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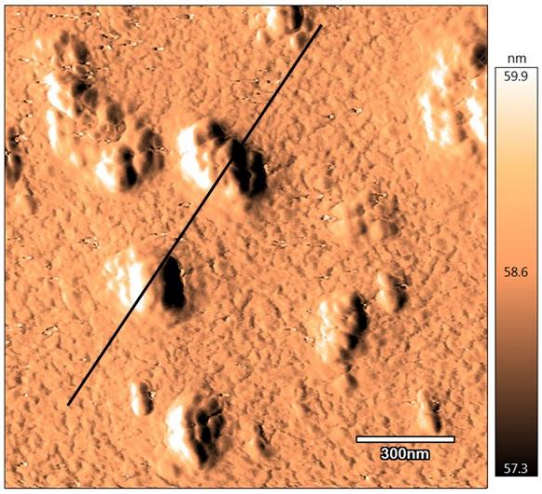

715 By Gaspard *et al.*

716 JDS.2019-17780 Figure 10

717

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718 **Figure 10.**

719 Atomic force microscopy images showing (A) phase, (B) height across the cross-section
720 marked in the 3D height image and (C) amplitude for a representative sample of 0.5% (w/v)
721 whey proteins and desialylated-CMP after heating at 80°C for 30 min at pH 6.JDS.2019-
722 17780 Figure 10

723

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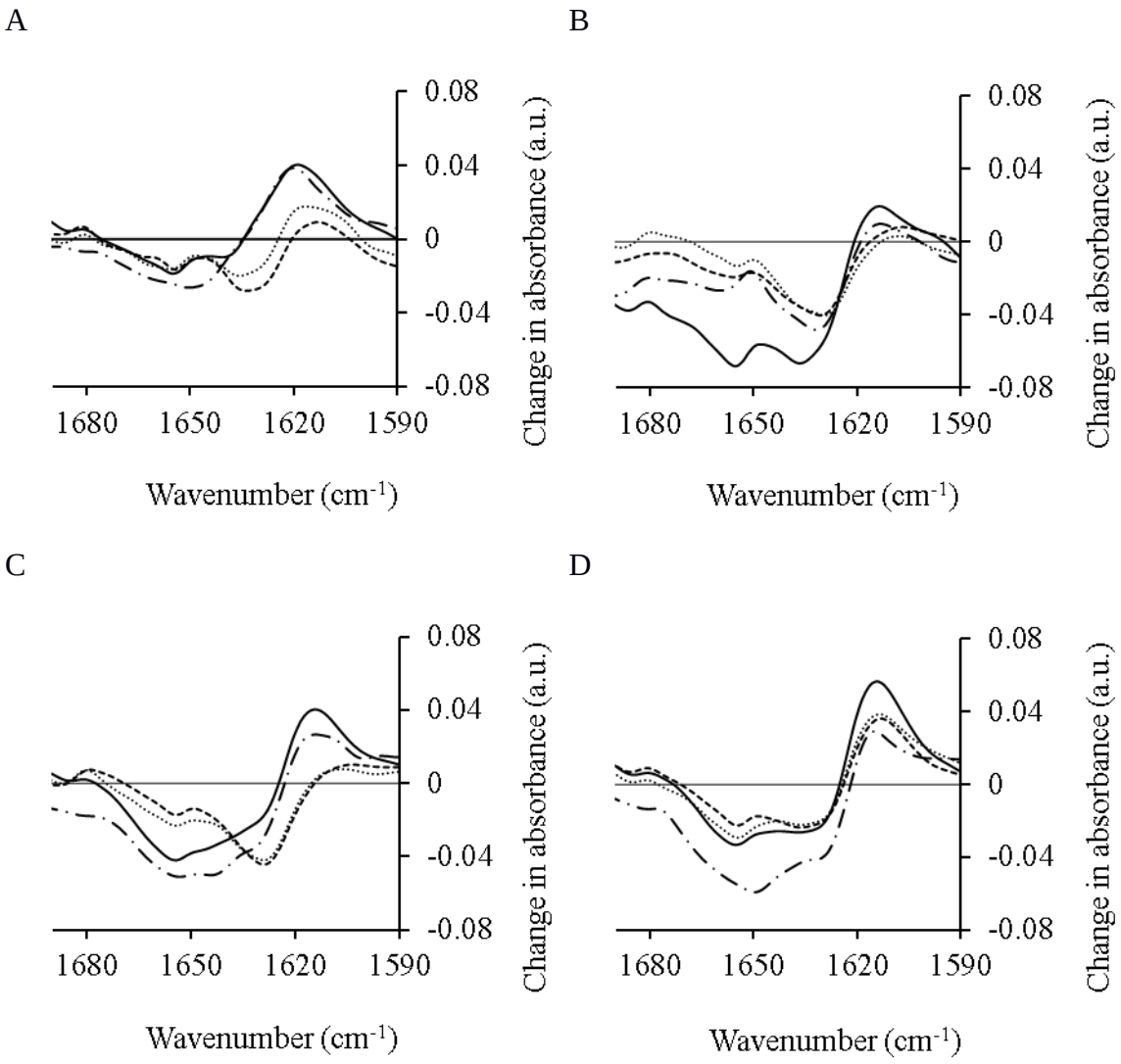

724 By Gaspard *et al.*

725 JDS.2019-17780 Figure 11

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Figure 11.

Attenuated total reflectance - Fourier transform infrared spectroscopy (ATR-FTIR) spectra of amide I bands of heated samples at (A) pH 4, (B) 5, (C) 6, (D) 7 containing (——) 5% (w/v) whey proteins (WP), (— .) 2.5% (w/v) WP, (- - -) 2.5% (w/v) WP with caseinomacropeptide (CMP) or (.....) 2.5% (w/v) WP with desialylated CMP (d-CMP), with the spectra of the corresponding non-heated samples substracted. The straight line through the origin highlighted the deviations in the spectra upon heating. The samples were heated at pH 4, 5, 6 or 7 for 30 min at 80°C and at pH 5 for 5 min. Experimental points were the average of data from two independent trials. The FTIR measurements were carried out on samples exhibiting some protein denaturation after heating for 30 min at 80°C. At pH 3, the whey proteins exhibited very little denaturation and aggregation after heating, therefore this condition was not tested here (Figure 4).

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