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FULL PAPER

Potential of whey protein isolate-lactose conjugates in the *in-vitro* infant digestion

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Abstract

Protein base modification is a notable potential method to alter the molecular structure and physicochemical and functional properties of the protein, thus affecting protein digestibility. This study investigated the protein digestibility of whey protein isolate–lactose (WPI-Lac) conjugates using an *in-vitro* infant gastric digestion static model. WPI was conjugated with lactose by dry Maillard reaction under optimised conditions. The following conditions were studied, WPI-Lac heated at 40°C, water activity a_w = 0.80 and incubation time of 0, 1, 3, 5 and 7 days. Based on the brown colour and the conjugation rates in *ortho*-phthaldehyde analysis, the incubation time of day 3 of conjugation promises the extent of conjugation and prevents the formation of advanced Maillard reaction products (MRPs). Functional properties of glycated protein were found to significantly higher ($p<0.05$) in antioxidant activity and solubility compared with native WPI. In addition, Fourier Transform Infrared (FTIR) analysis indicated that the WPI was modified by dry MR with lactose. WPI-Lac day 3 was evaluated using the *in-vitro* gastric infant digestion model which undergo simulated gastric infant condition at pH 3 with 19 µL of 0.625mg/mL of pepsin. Sodium dodecyl sulfate acrylamide gel electrophoresis (SDS-PAGE) analysis of digesta revealed that MRPs increased susceptibility to be hydrolysed by pepsin. The digestion product affirms dry MR conjugation can potentially improve WPI digestibility. Herein, this study of WPI-Lac conjugates contributes to an understanding of how protein–disaccharide glycates affect *in-vitro* infant gastric digestion.

1. Introduction

Human milk (HM) comprises hundreds of proteins with a wide range of roles that are likely to lead to breastfeeding's short- and long-term benefits (Donovan, 2019). In addition, these digestible proteins provide a well-balanced source of amino acids for the swift development of infants (Lönnerdal, 2003). Whey protein and casein are found in HM proteins (Ballard and Morrow, 2013; Chatterton *et al.,* 2013; Tu *et al.,* 2014; Nguyen *et al.,* 2015; Halabi *et al.,* 2020). However, whey protein profiles in HM do not contain β-lactoglobulin (β-Lac), leading to some disparity with infant formulas (Halabi *et al*., 2020). Infant formulas are based on cow's milk (Maathuis *et al.,* 2017; Golkar *et al.,* 2019; Ye *et al.,* 2019). The high percentage of β-Lac in cow's milk (i.e., 80%) (Donovan, 2019; Ye *et al.,* 2019) could trigger allergies in infants (Chatterton *et al.,* 2013). Given the isoelectric protein (pI) of β-Lac (pI 5.2) (Damodaran, 2007) this protein is highly soluble because of the large ratio of surface hydrophilic residues to surface nonpolar groups but relative resistance to acid hydrolysis (Wal, 2004). In 2018, resistance to gastrointestinal digestion appeared to be one of the main characteristics of proteins that trigger an allergic response via the gastrointestinal tract (Pekar *et al.,* 2018). Food protein digestion is complex and critical in infants. Given the growing realisation that digestion in infants is different from that in adults, investigations on this process have broadened through the use of the *in-vitro* digestion model. Thus, the *in-vitro* digestion models have received much attention.

Although *in-vitro* models do not fully mimic *in-vivo* conditions, they have been widely used because they have no ethical constraints, have low cost and can be easily prepared for sampling (Nguyen *et al.,* 2015; Nguyen, 2017; Gan *et al.,* 2018; Ménard *et al.,* 2018). Meanwhile, Shani-Levi *et al*. (2017) presented an overview of some new and emerging *in-vitro* digestion models that mirror the gastrointestinal conditions of infants and mentioned that these models showed

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advantages, such as rapidity, and less labour intensive and having significantly fewer bioethical constraints. Understanding food digestion in the human gastrointestinal tract greatly affects the food industry, which is expected to produce nutritious content in food (e.g. proteins, carbohydrates and fats). Hur *et al*. (2011) reported that understanding the digestibility, structural changes and kinetics of food under closely simulated physiological conditions in the human gastrointestinal tract requires the application of *in-vitro* models. Previous research has investigated the digestion of proteins using *in-vivo* models, results demonstrated full complex digestion but concerns about time, expense and ethical constraints, particularly when considering an infant. Fortunately, *in-vitro* models are built to mimic physiological *in-vivo* conditions, which consist of complex systems, as reported in Coles *et al*. (2005), including the composition of and subsequent digestive secretion, digestion and absorption and the interaction between the host, the food and microbacteria in the digestive system. *In-vitro* digestion models are preferred method to investigate food digestion in humans because of their versatility, reproducibility, time efficiency, cost-effectiveness and most importantly the absence of ethical restrictions (Picariello *et al.,* 2013; Minekus *et al.,* 2014).

Recently, Egger *et al*. (2019) evaluated the comparison of protein digestion using *in-vitro* static and dynamic protocols. They found out that the *in-vitro* dynamic protocol reflected the improved mimicking conditions of *in-vivo* analysis showing a good approximation of peptide patterns in the gastric and intestinal endpoints. As mentioned in Ménard *et al.* (2014), this sophisticated model includes pH regulation, dynamic food flows and digestive enzyme concentration, by contrast, *in-vitro* dynamic digestion models require complex operations and huge costs for setting up the equipment and the system. By contrast, Egger *et al.* (2019) mentioned that the dynamic protocol considerably reflected physiological *in-vivo* conditions at the level of free amino acid release. However, in Egger *et al*. (2019), the absorption of free amino acids was not imitated in both *in-vitro* digestion protocols, thereby indicating restraints in mimicking *in-vivo* conditions. This finding was supported by Hur *et al.* (2011), Coles *et al*. (2005), and Lucas-González *et al*. (2018). It has some significant implications for the digestion process of protein *in-vitro*. It concerns the differences in digestibility among infants of different ages, especially during their feeding development stages. The composition of the food consumed by infants is constantly changing over time and it is not possible to mimic this complexity with an *in-vitro* analysis.

One of the methods to modify base proteins is the Maillard reaction (MR). MR refers to a reaction of protein and sugar via heat processing without the presence of enzymes. Teodorowicz *et al*. (2017) explained that during MR, covalent bonds are formed when proteins and sugar are linked with the free amino groups of amino acids (mostly lysine and the carbonyl groups of the reducing sugar). However, MR proteins with polysaccharides do not react with high reactivity as fast monosaccharides and disaccharides do; thus, at the initial stage of the MR pathway, the reaction is stopped, yielding a high concentration of Schiff base (Zhu *et al.,* 2008; Oliveira *et al.,* 2016). By contrast, Fu *et al*. (2002) reported that no direct connection exists between digestibility measured *in vitro* and allergenicity. Eleven years later, Böttger *et al*. (2013) investigated the relationship of digestibility on modified proteins with *in-vitro* digestion. They found that β-Lg is digested in glycates, but small amounts of glycates and β-Lg are not fully digested at the end of duodenal digestion. Interestingly, modified proteins successfully mask immunogenic peptides by dextran, but the digestion kinetic for β-Lg present in glycates occur at a somewhat slower rate because dextran provides some steric hindrance (Böttger *et al.,* 2013).

Despite the importance of MR in protein modification, information about the conjugation of proteins with disaccharide sugar of lactose remains lacking. In addition, the digestibility of the conjugated whey proteins with lactose must be explored to understand the modified molecular structure, as well as their bioaccessibility in gastrointestinal tracts in infants. Such investigation could help in the development of hypoallergenic food proteins and the masking of immunogenic peptides by lactose. To date, many researchers have succeeded in modifying native proteins. The creation of a new generation of functional whey protein-based ingredients may benefit from the modification of whey protein functionality through Maillard-induced glycation. Previous research reported the types of amino groups and sugars that act as reducing and non-reducing sugar, affect MR products. Some attempts have been made to improve the functional properties of enzymes and food proteins by conjugation with glucose-6-phosphate (Kato *et al*., 1995) or polysaccharides such as dextran and galactomannan (Kato *et al.,* 1988; Kato *et al.,* 1992) through the MR. Chevalier *et al.* (2001) studied the MR to improve the functional properties (solubility, heat stability, emulsifying and foaming properties) of β-lactoglobulin (β-LG) glycated with several sugars (arabinose, galactose, glucose, lactose, rhamnose or ribose), whereas Li *et al.* (2011) investigate on the effect of MR conditions (weight ratio of protein to sugar, temperature and time) on the antigenicity of α-lactalbumin (α-LA)

and β-lactoglobulin (β-LG) in conjugates of whey protein isolate (WPI) with maltose. While de Oliveira *et al.* (2016) use polysaccharides conjugated with protein in their research. Covalent bonds form when protein conjugates to carbohydrates, which undergo a complex chemical alteration during MR reaction. Glycated proteins with carbohydrates play a vital role in the modification of the physicochemical and functional properties of the protein. In addition, the conjugation of proteins with carbohydrates via MR enhances the nutritional properties of the protein, resulting in a growing interest to investigate conjugated proteins (Zhang *et al.,* 2019). Substantial research has discussed the conjugation of proteins with sugar; however, studies on the effect of lactose on the antigenicity of β-Lac in whey proteins through dry MR during *in-vitro* infant digestion are scarce. Therefore, studying the conjugated protein glycates with lactose via *in-vitro* static protocol in the gastrointestinal tract of infants may further enhance the knowledge and understanding of the effects of glycated protein–lactose in protein digestion amongst infants and the correlation of these glycated proteins with immunogenic protein. Therefore, this study could help the production of modified proteins with optimised conditions towards bioaccessibility and absorption benefits for infants. Moreover, this research was aimed to determine the physicochemical and functional properties of conjugated whey protein–lactose and analyse the potential of the conjugated protein–lactose in *in-vitro* infant digestibility via a static method.

2. Materials and methods

2.1 Materials

Whey protein isolate (WPI) was obtained from Nutrija Lifesciences, India. Lactose (Lac) (Ph. Eur, Sigma-Aldrich/US) was purchased from Weitengen Sdn Bhd, MAS. All other chemicals and reagents used were analytical grade.

2.2 WPI-Lac conjugate preparation

The conjugation of protein and lactose was performed in accordance with the method of Julmohammad (2017). WPI and lactose were dissolved in 40 mL of purified water with a protein-to-sugar ratio of 0.4:1 (wt/wt). The mixtures were then frozen for 24 hrs and lyophilised (Labconco, 4.5 L, -50°C, Kansas City, USA) to obtain a dry powder. Subsequently, the powder was incubated at 40°C in a desiccator containing a saturated KBr solution with 0.8 water activity, a_w (Wexler, 1994). Samples were taken periodically after 0, 1, 3, 5 and 7 days of incubation for further characterisation.

2.3 Colour observation

The colour observation was performed using the method of Liu *et al*. (2019), 2 and 10 mg/mL of WPI or WPI-Lac were measured at 294 and 420 nm absorbance, respectively, using an ultraviolet-visible (UV-Vis) spectrophotometer (PerkinElmer Lambda, US).

2.4 L a* b* analysis*

WPI or WPI-Lac were placed into a 5 cm diameter glass petri dish. Three colour components, namely, L* (black–white, luminosity), a* (+red to −green) and b* (+yellow to −blue), were recorded using a Minolta chroma meter (Morales and Van Boekel, 1998).

2.5 SDS-PAGE analysis

SDS-PAGE was performed using 15% of resolving gel and 4% stacking gel in accordance with the method described by Laemmli (1970), with the following modifications: Ten microlitres of protein sample at 1.0 mg/mL were dissolved with 10 µL of SDS sample buffer under reducing condition (4%–20% Tris-glycine SDS-PAGE) and then heated to 90°C for 10 mins. Aliquots of 5 µL of the sample were loaded into each well on the gel. Protein markers with ~10 to ~250 kDa molecular weight standard protein were also run as indicators for the sample, and electrophoresis was conducted at 180 V minimum an hour. After electrophoresis, the gel was stained using a Coomassie blue R-250 kit (Bio-Rad, US).

2.6 Ortho-phthaldehyde *analysis*

Samples were pipetted into a 96-well clear plate, and 200 µL of OPA reagent was added, with absorbance at 340 nm. The degree of glycation was determined using the equation below:

$$\text{Degree of glycation (\%)}: \frac{A_0 - A_t}{A_0} \times 100$$

Where A_0 is the absorbance of control without sample, and A_t is the absorbance of the sample. All samples were analysed in triplicate (Chevalier *et al.*, 2001).

2.7 FTIR analysis

Spectroscopic measurements were performed using the method of Liu *et al*. (2014) with minor modifications. A total of 25 mg of the sample was mixed with 225 mg of KBr (10% w/w) and then measured in transmission mode from 4000 cm^{-1} to 400 cm^{-1} at room temperature.

2.8 Solubility

Approximately 2 g of the sample was dissolved in 100 mL of demineralised water, and 1 hr was needed for the equilibration time of the samples before pH 5 was set

using 1 M NaOH or HCl. Samples were then centrifuged at 4000 rpm for 90 mins (Wefers *et al.,* 2018).

2.9 Antioxidant properties

Twenty microlitres of the samples at 10 mg/mL concentration after 0, 1, 3, 5 and 7 days of incubation were dissolved with diluted 2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) solution. The mixture was incubated in the dark for 6 mins and measured with an absorbance of 0.70±0.01 at 734 nm. ABTS radical scavenging activity was measured in percentage (%) using the equation below:

$$\text{ABTS radical scavenging activity (\%)}: \frac{A_c - A_s}{A_c} \times 100$$

Where A_c is the absorbance of the control, and A_s is the absorbance of the sample. All samples were analysed in triplicate (Liu *et al.,* 2014).

2.10 Simulated gastric fluid

The simulated gastric fluid (SGF) solution was prepared in accordance with the method of Böttger *et al.* (2013). In addition, 0.15 M of NaCl solution with pH 3.0. HCl was used to adjust the SGF pH solution.

2.11 In-vitro gastric digestion

In-vitro gastric digestion was performed in accordance with the method of Böttger *et al.* (2013), with minor modification. The WPI and glycate solution had a concentration of 4 mg/mL and a volume of 1.5 mL. The pH of the sample solution was adjusted to pH 3 using 0.5 M HCl, and the final volume of the sample solution was then brought up to 1.98 mL using SGF. Nineteen microlitres of 0.625 mg/mL of pepsin stock solution were added into the sample solution to a final protein concentration of 3 mg/mL and then placed into an Eppendorf tube. The temperature was set at 37°C, and 200 rpm was used to start the digestion process. As for the control, 100 µL was taken out before digestion started, and 100 µL of aliquots were directly pipetted into 20 µL of ammonium bicarbonate (NH_3CO_3) after the digestion process time was set to 5, 30 and 60 mins. Ice was placed immediately on digesta and stored at −20°C. Gastric reminder containing pepsin was inactivated by increasing the pH to 7 in 10 mins.

2.12 SDS-PAGE digesta

SDS-PAGE digesta was performed under reducing conditions on a Mini-Protean 3 cell from Bio-Rad (Mini-Protean TGX Precast Gel (Tris-HCl gel, 4-20% linear-gradient, 15 wells, 456-1096; Bio-Rad). Electrophoresis was performed at 200 V and 22°C for 35 mins. Prestained Rec Protein Ladder (BP3603, Fisher Scientific) 1-170KDa and gels were stained using Coomassie blue G-250 (161-0786, Bio-Rad) as described in the method of Böttger *et al.* (2013).

2.13 Statistical analysis

Statistical analysis was determined using IBM SPSS statistics software (Version 27, IBM). One-way analysis of variance (ANOVA) was used for the comparison of mean values and further separated by Duncan significant difference test. All data were reported as means ± standard deviation (SD).

3. Results and discussion

3.1 Colour observation

The absorbance at 294 nm indicator of colourless intermediate products in MR (Corzo-Martínez *et al.,* 2014) cited by (Nooshkam and Madadlou, 2016), whereas at 420 nm indicator of advanced stages of MRPs (Chen *et al.,* 2019; Zhang *et al.,* 2020). Figure 1 shows that at 294 nm, WPI native had an increasingly significant difference ($p<0.05$), whereas the MPRs of WPI-Lac at 294 nm decreased during the early incubation days (from day 0 to 5) in Table 1, such decrease could be attributed to the few available carbonyls and free amino groups when the days of incubation was longer and then increase significantly ($p<0.05$) in day 7 due to aggregation of end-stage of MPRs (Chen *et al*., 2019) which was different from Nooshkam and Madadlou (2016) finding on the conjugation of WPs with lactose. This result might be due to the contrast in the reaction mechanism. The increase of 294 nm values indicated the formation of an uncoloured compound which could be the precursor to the formation of browning colour pigment (Huang *et al.,* 2012). The important characteristics of MR include the formation of brown MPRs (Liu *et al.,* 2019), thus advanced brown colour is observed by measuring the absorbance at 420 nm to measure the advance of MR. As observed in Figure 2 the absorbance values at 420 nm of WPI native and WPI-Lac decreased and then increased with incubation days. This result indicates that from day 1 to day 5, the intermolecular reaction might occur between the WP and the sugar for WPI Native and WPI-Lac; as a result, the availability of free amino groups and carbonyl for conjugation was limited. On day 7 in Table 2, absorbance increased due to the formation of melanoidins, which were brown. Brown colour was observed in the MR of WPI with different types of sugar. A higher intensity of brown colour was observed at longer incubation days; this result is consistent with that of Corzo-Martínez *et al.* (2014), Nooshkam and Madadlou (2016), Santos *et al.* (2019), Chen *et al.* (2019) and Zhang *et al.* (2020).

Table 1. Colour observation of WPI and WPI-Lac at absorbance of 294 nm.

Days	WPI Native	WPI-Lac
0	0.9708±0.0020^{c}	0.8453±0.0000^{e}
1	0.9085±0.0054^{a}	0.7626±0.0004^{a}
3	0.9216±0.0064^{b}	0.7846±0.0001^{c}
5	0.9122±0.0019^{ab}	0.7720±0.0006^{b}
7	0.9623±0.0004^{c}	0.8016±0.0001^{d}

Values are presented as mean±SD. Values with different superscript within the same row are significantly different by the Duncan's test ($p<0.05$).

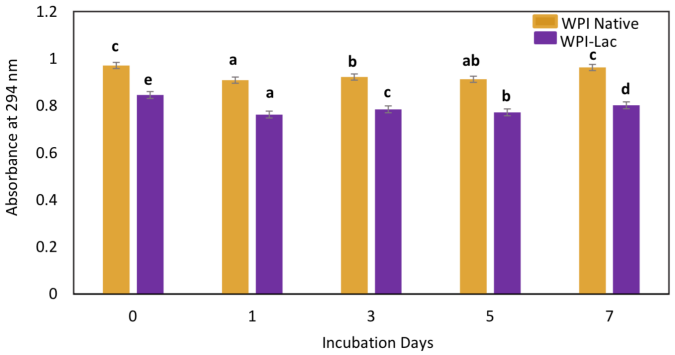

Figure 1. Colour observation of native protein and intermediate stage of MRPs at absorbance at 294 nm. Bars with different alphabet notations are significantly different ($p<0.05$) by the Duncan's test.

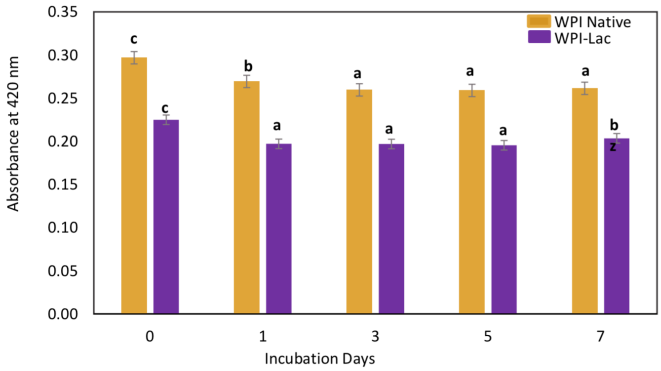

Figure 2. Colour observation of native protein and an advanced stage of MRPs at absorbance at 420 nm. Bars with different alphabet notations are significantly different ($p<0.05$) by the Duncan's test.

3.2 L a* b* analysis*

The browning degree of each conjugated protein–sugar sample was determined via L* a* b* analysis. Colour L (lightness) is used to evaluate the degree of the brown colour of MRPs. As shown in Table 3, WPI-Lac decrease significantly different ($p<0.05$) from 89.127 to 84.930 with the agreement of longer incubation time result in increasing brown colour pigment on the sample and indicate that Lac greatly influenced the colour of glycates, while WPI Native shown L* values were higher in day 7 (Figure 3) indicates showing no conjugation had occurred. For a*, measure red or green value of glycates, WPI Native in Figure 4 is shown to decrease significantly ($p<0.05$), whereas glycated protein

Table 2. Colour observation of WPI and WPI-Lac at absorbance of 420 nm.

Days	WPI Native	WPI-Lac
0	0.2969±0.003^{c}	0.2250±0.001^{c}
1	0.2694±0.001^{b}	0.1971±0.003^{a}
3	0.2596±0.003^{a}	0.1969±0.000^{a}
5	0.2590±0.000^{a}	0.1954±0.000^{a}
7	0.2614±0.000^{a}	0.2035±0.004^{b}

Values are presented as mean±SD. Values with different superscript within the same row are significantly different by the Duncan's test ($p<0.05$).

increases significantly different ($p<0.05$) from 0.517 to 1.303 (Table 4). This indicates advanced MR occur during incubation day 7 of glycates. Figure 4 depicts that at day 3, glycated protein slightly decreased in values probably due to the aggregation of amino proteins because the degree of conjugation was lower than that of other incubation days. Figure 5 shown slightly increase significantly different ($p<0.05$) of WPI native in b* values, and WPI-Lac values were increase significantly different ($p<0.05$) with incubation time from 10.103 to 17.157 (Table 5). As protein was incubated extensively (day 7), the brown colour of MRPs intensified, thus gaining attention to health and low usage potential in food ingredients (Nooshkam and Madadlou, 2016).

Table 3. L* values from non-conjugated and conjugated protein at a_w 0.79, 40°C for 0, 1, 3, 5 and 7 days.

Days	WPI Native	WPI-Lac
0	87.263±0.01^{b}	89.127±0.01^{d}
1	86.967±0.01^{a}	88.793±0.01^{c}
3	89.310±0.00^{c}	88.563±0.01^{b}
5	89.727±0.01^{d}	89.130±0.00^{d}
7	90.663±0.01^{e}	84.930±0.01^{a}

Values are presented as mean±SD. Values with different superscript within the same row are significantly different by the Duncan's test ($p<0.05$).

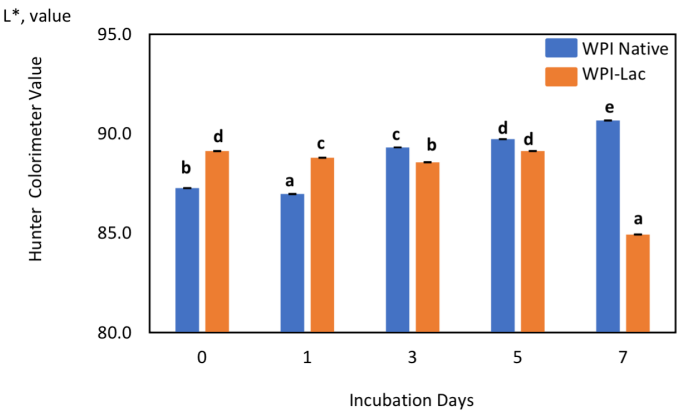

Figure 3. L* values from non-conjugated and conjugated protein at a_w 0.79, 40°C for 0,1,3,5 and 7 days. Bars with different alphabet notations are significantly different ($p<0.05$) by the Duncan's test.

Table 4. a* values from non-conjugated and conjugated protein at a_w 0.79, 40°C for 0, 1, 3, 5 and 7 days.

Days	WPI Native	WPI-Lac
0	0.253±0.01^{d}	0.517±0.02^{c}
1	0.167±0.01^{c}	0.483±0.02^{b}
3	0.100±0.02^{b}	0.263±0.02^{a}
5	0.173±0.02^{c}	0.640±0.01^{d}
7	0.033±0.01^{a}	1.303±0.01^{e}

Values are presented as mean±SD. Values with different superscript within the same row are significantly different by the Duncan's test ($p<0.05$).

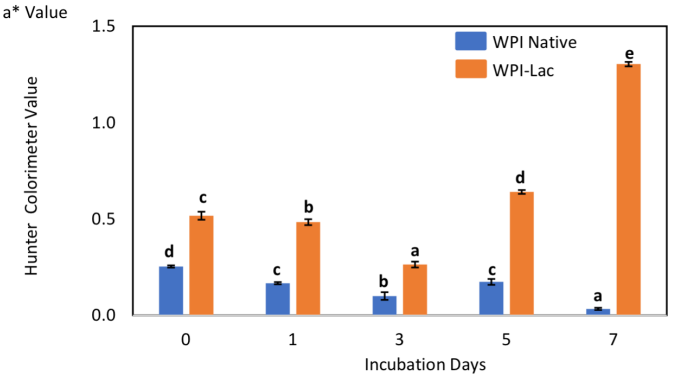

Figure 4. a* values from non-conjugated and conjugated protein at a_w 0.79, 40°C for 0,1,3,5 and 7 days. Bars with different alphabet notations are significantly different ($p<0.05$) by the Duncan's test.

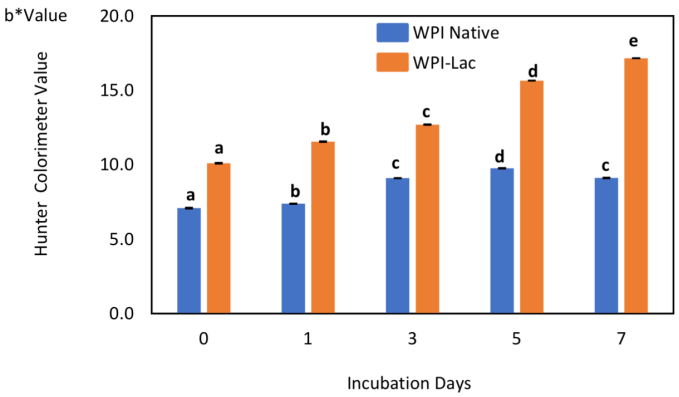

Figure 5. b* values from non-conjugated and conjugated protein at a_w 0.79, 40°C for 0,1,3,5 and 7 days. Bars with different alphabet notations are significantly different ($p<0.05$) by the Duncan's test.

3.3 SDS-PAGE analysis

Figure 6 and Figure 7 show an increase in the intensity of conjugated bands, with a formation of higher molecular weight species in lanes 4–6. The bands slightly shifted upward around 20 kDa due to the increase in MW, indicating that the conjugation of WPI and Lac occurred. Lanes 1–3, which are visible at ~15 kDa, indicated no covalent interaction, which affects the SDS-PAGE profile compared with conjugated protein. SDS-PAGE was determined the qualitative analysis, therefore, to provide quantitative analysis on the correlation of the conjugation of WPI and Lac, OPA analysis was used, as described in the next subpoint.

Table 5. b* values from non-conjugated and conjugated protein at a_w 0.79, 40°C for 0, 1, 3, 5 and 7 days.

Days	WPI Native	WPI-Lac
0	7.087±0.03^{a}	10.103±0.03^{a}
1	7.380±0.01^{b}	11.553±0.03^{b}
3	9.097±0.01^{c}	12.697±0.02^{c}
5	9.763±0.02^{d}	15.653±0.01^{d}
7	9.113±0.02^{c}	17.157±0.01^{e}

Values are presented as mean±SD. Values with different superscript within the same row are significantly different by the Duncan's test ($p<0.05$).

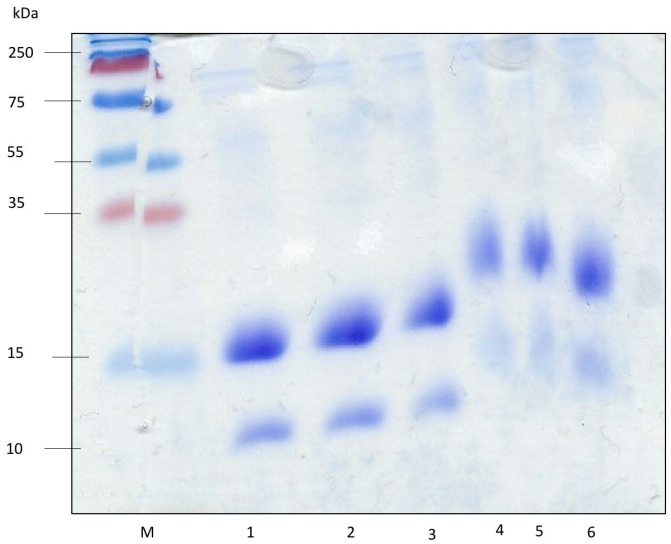

Figure 6. SDS-PAGE pattern for native protein and conjugated protein. Lane M: Marker standard, Lane 1: WPI Day 0, Lane 2: WPI Day 1, Lane 3: WPI Day 3, Lane 4: WPI-Lac Day 3, Lane 5: WPI-Lac Day 1 and Lane 6: WPI-Lac Day 0.

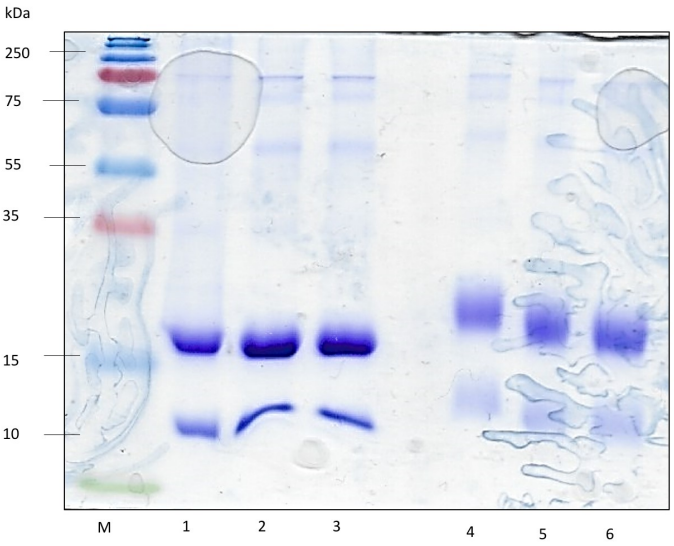

Figure 7. SDS-PAGE pattern for native protein and conjugated protein. Lane M: Protein marker (standard), Lane 1: WPI Day 3, Lane 2: WPI Day 5, Lane 3: WPI Day 7, Lane 4: WPI-Lac Day 7, Lane 5: WPI-Lac Day 5 and Lane 6: WPI-Lac Day 3.

3.4 Ortho-phthaldehyde analysis

To determine the extent of glycosylation by a degree of conjugation, the *ortho*-phthaldehyde (OPA) method can be used by measuring the contents of free amino groups in the conjugated and native proteins (Boostani *et al.,* 2017; Zhang *et al.,* 2019). The number of free amino groups is expressed as the percentage of protein to the total free amino groups, as shown in Figure 8, from incubation days 0 to 7. WPI native showed a slight decrease in the percentage of free amino groups available with incubation time (Table 6). This result may be due to the lactosylation brought about by the presence of lactose in these samples. As expected, conjugated protein showed a decrease in free amino groups in the sample on -site. Dry MR enhances the protein to unfold as a result the cleavage sites of protein were accessible (Wada and Lönnerdal, 2014; Mulet-Cabero *et al.,* 2019), thus lactose was more reactive with the WPI.

Table 6. OPA assay expressed as percentage free amino groups of WPI Native and WPI-Lac, as a function of incubation time (days) at 40°C

Sample	Incubation Days	Mean± SD	n/b Site (%)
WPI Native	0	0.3985±0.04	100
	1	0.3942±0.03	99
	3	0.3862±0.03	96
	5	0.3660±0.01	90
	7	0.3745±0.03	93
WPI-Lac	0	0.3186±0.03	100
	1	0.2893±0.01	89
	3	0.2503±0.01	75
	5	0.2357±0.00	69
	7	0.2233±0.01	65

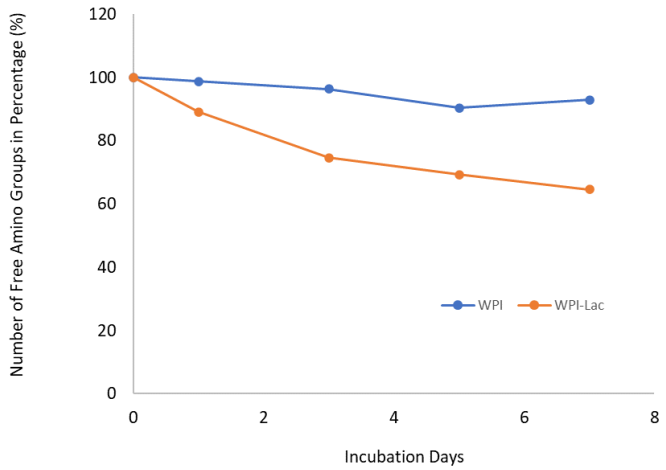

Figure 8. OPA assay expressed as percentage free amino groups of WPI Native and WPI-Lac, as a function of incubation time (days) at 40°C.

3.5 FTIR analysis

FTIR technique is determined by the absorption of radiation, which is due to the vibration of atoms in molecules (Liu *et al.,* 2014). This method yields information of functional group region in the FTIR graph and the "fingerprint" region, which can be used to identify the sample, whereas FTIR spectroscopy can be used to provide information about the structure and interactions of polysaccharides and proteins (Su *et al*., 2010). Proteins conjugating with disaccharide sugar leads to a new molecular structure with novel functional properties; this finding is supported by Deng *et al.* (2020), who reported that *in-vitro* gastric digestibility is higher when the protein structure is modified. As observed in Figure 10 conjugated proteins become more intense from 3000 cm^{-1} to 3500 cm^{-1}. Broad peak as seen in WPI-Lac day 5 (Figure 10), caused by –OH stretching. The sugar band region from 1180cm^{-1} to 953 cm^{-1} (Hernández-García *et al.,* 2016) showed overlapping peaks, indicating the vibration of C–C and C –O stretching and C–H bending. WPI native showed changes in the protein band COO– region from 1700 cm^{-1} to 1500 cm^{-1} Figure 9 which is different from sharp bands in glycated protein. At absorption 1100 cm $^{-1}$ to 1000 cm $^{-1}$, glycated protein showed decreasing intensity, this might be loss of the functional groups, such as N–H, indicated conjugation had occurred. Protein structural changes in samples due to the conjugation would enhance the accessibility of hydrophobic structures in protein. This change increases the hydrolysis in *in-vitro* gastric digestion by pepsin (Ahn *et al.,* 2013). Thus, peptic cleavage sites would be accessible in gastric digestion. In the presence of lactose, dry conjugation of BLG in samples lead to higher peptic hydrolysis (Chevalier *et al.,* 2002) and this finding is supported by Böttger *et al.* (2013).

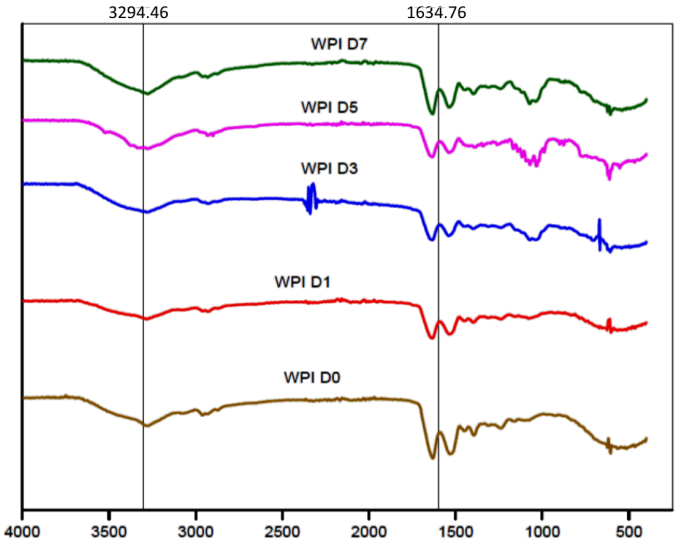

Figure 9. FTIR spectra of WPI native from day 0 to day 7 respectively. The measure in transmission mode from 4000 cm−1 to 400 cm−1 at room temperature.

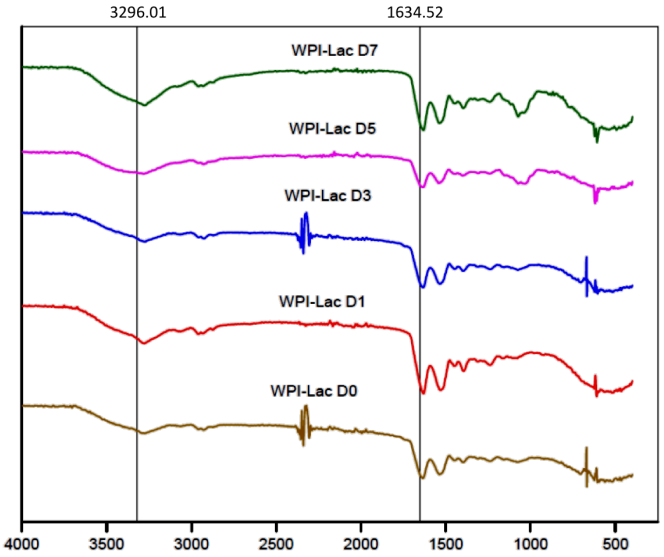

Figure 10. FTIR spectra of WPI-Lac from day 0 to day 7 respectively. The measure in transmission mode from 4000 cm−1 to 400 cm−1 at room temperature

3.6 Solubility

The increased polarity of WPI-Lac indicates higher solubility glycates due to a covalent linkage with the hydrophilic functional group of Lac (–OH), which occurs in di-OH in Lac. As a result, aggregation was inhibited (Chen *et al*., 2019). Figure 11 shows the increased values for WPI-Lac with a longer incubation time from day 0 to day 3. This finding is consistent with that of Yu *et al.* (2020). Day 5 to day 7 of WPI-Lac showed a decrease due to the aggregation of protein molecules, which can reduce the hydrophilic site on the protein surface to react with the active site of Lac (Yu *et al.,* 2020). Wafers *et al.* (2018) showed that longer incubation time results in the poor solubility of native proteins. The formation of covalent bonds between protein and reducing sugar via MR provides advantages in terms of increasing the solubility of conjugated protein when glycosylation analysis was performed by Yu *et al.* (2020) using peanut protein and lactose. In addition, the solubility of BLG conjugate with fructo-oligosaccharides significantly increased (Zhong *et al.,* 2013). In *in-vitro* digestion, protein becomes more digestible when the structure is lost and unfolded, thereby increasing the accessibility of proteases in the active sites of protein (Verhoeckx *et al.,* 2019). Dry heating protein can cause proteins to unfold, unfortunately increasing surface hydrophobicity, which leads to aggregation. Thus, an alternative dry MR of protein with sugar would help. Previous research has reported that the BLG is resistant to pepsin in gastric digestion, but researchers have proven that the heating of protein with dextran (Böttger *et al.,* 2013) increases the susceptibility to pepsin hydrolysis. Whereas, in 2014, Tu *et al.* showed the ability of milk protein conjugates with lactose a higher digestibility in *in-vitro* digestion compared to the native sample. They indicated that high solubility obtains high digestibility, this finding could benefit the milk protein industry.

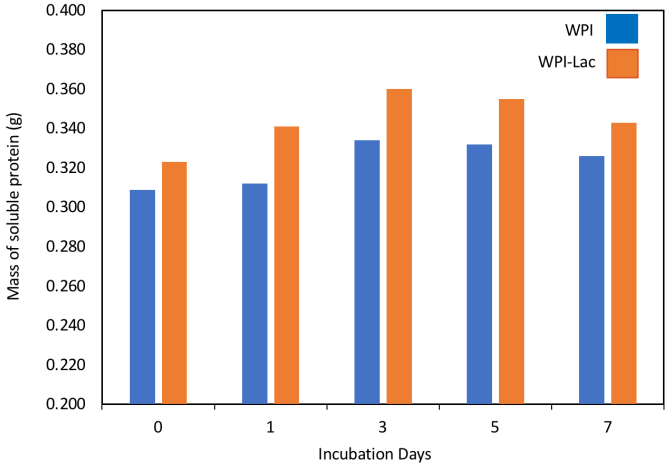

Figure 11. The solubility of WPI Native and WPI-Lac with function of incubation days.

3.7 Antioxidant activity

Longer periods of incubation time increase the glycation degree of the sample, causing an intermediate and dark brownish formed MPRs and resulting in the higher molecular mass of sample (Nooshkam *et al.,* 2019) as determined in shifted upward band in SDS-PAGE Figure 6 and Figure 7. The high browning intensity of the sample shows good antioxidant activity. Table 7 depicted greater ABTS radical scavenging activity in conjugated protein. The longer incubation time, the higher percentage significantly different ($p<0.05$) from 10% to 62% (Figure 13). The native protein showed an increase on day 7 probably due to the presence of lactose in the sample Figure 12. Thus, heating time with sugar present increases the intensity of the brownish colour of the sample, resulting in higher ABTS activity in the sample. This result is consistent with that of Liu *et al.* (2014) and Nooshkam *et al.* (2019). Tu *et al.* (2014) showed the Trolox equivalent

Table 7. ABTS Radical Scavenging of WPI Native and WPI-Lac at 10.00 mg/mL.

Sample	Incubation Days	Mean± SD
WPI Native	0	0.6911 ± 0.00^{c}
	1	0.6848 ± 0.00^{bc}
	3	0.6865 ± 0.00^{bc}
	5	0.6820 ± 0.00^{b}
	7	0.6547 ± 0.01^{a}
WPI-Lac	0	0.6434 ± 0.01^{e}
	1	0.5387 ± 0.01^{d}
	3	0.4767 ± 0.01^{c}
	5	0.3739 ± 0.01^{b}
	7	0.2725 ± 0.01^{a}

Values are presented as mean±SD. Values with different superscript within the same row are significantly different by the Duncan's test ($p<0.05$).

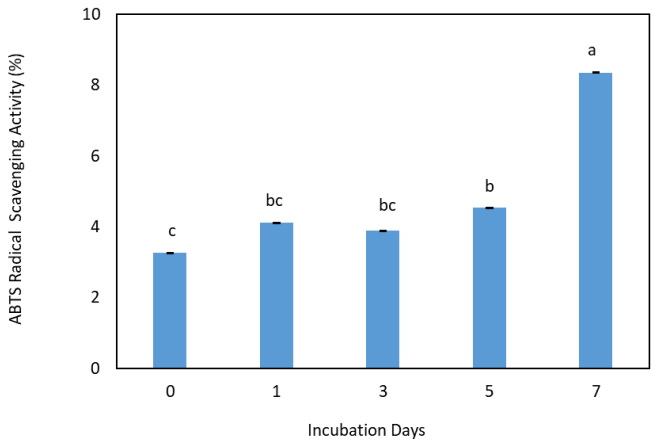

Figure 12. ABTS Radical Scavenging of WPI Native at 10.00 mg/mL. Bars with different alphabet notations are significantly different ($p<0.05$)

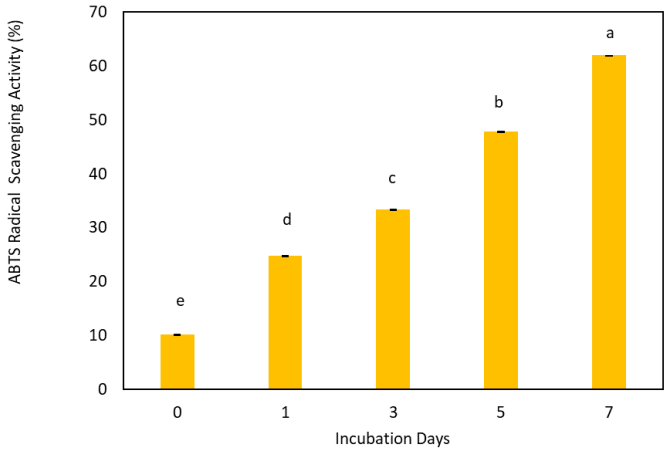

Figure 13. ABTS Radical Scavenging of WPI-Lac at 10.00 mg/mL. Bars with different alphabet notations are significantly different ($p<0.05$).

antioxidant capacity (TEAC) of milk protein-lactose conjugates digests increased, indicating that the degree of hydrolysis increases during *in-vitro* digestion. Thermally treated samples improve the hydrophilicity of digesta and enhance the ABTS radical scavenging activity. Conjugated proteins with carbohydrates have shown high levels of antioxidant activity, which may lead to interest as an antioxidant in the formulated food system (Jiang and Brodkorb, 2012).

3.8 SDS-PAGE digesta

A systematic understanding of the mechanism of protein digestion in infants can be revealed by the gastrointestinal tract. The *in-vitro* model is proposed as an alternative to *in-vivo* experiments. A research finding by Hur *et al.* (2011) also points towards that understanding the digestibility, structural changes and kinetics of food under closely simulated physiological conditions in the human gastrointestinal tract, *in-vitro* models have been applied. Researchers have performed extensive experiments on the infant digestion process through *in-vitro* gastrointestinal models, even though *in-vitro* studies on physiological procedures on the infant digestive system have been spurned for the last decade (Gan *et al*., 2018).

An investigation has been conducted using these *in-vitro* models to examine and analyse the allergenicity of protein amongst infants. This current study was chosen as an infant digestion model because cow's milk protein allergy (CMA) has been identified as a major contributing factor of allergenicity for a particular population of infants. CMA has been intensively investigated due to its allergic effects on infants (Crittenden and Bennett, 2005). This finding is supported by Meyer *et al*. (2018), who revealed that CMA is the most notable food allergy in children, and it generally ranges between 1.9% and 4.9% at age less than 1 year. On the other hand, Høst (2002) claimed that CMA is most notable in infancy and shows a significant percentage of approximately 2% to 3% of children aged 0–3 years. In 2018, Moimaz *et al.* published a paper in which they described for the past 15 years various investigations on the nomenclature of allergic reactions to milk and diagnosis has been made. Furthermore, Pekar *et al.* (2018) mentioned that an allergic response triggered by protein through the gastrointestinal tract is likely resistant to gastrointestinal digestion.

This study focused on MRPs based on lactose in infant *in-vitro* gastric digestion. The proteolysis of WPI as control and WPI-Lac glycates were analysed from SDS-PAGE under reducing conditions (Figure 14). During the gastric digestion of WPI-Lac, ALA increased susceptibility hydrolysed by pepsin. The isoelectric point of (*pI*) ALA is 4.2. According to Damodaran *et al.* (2007), state that proteins at their (*pI*) are more stable against denaturation. When a high net charge of extreme acid pH values is resulting in unfolding proteins, however, ALA has been investigated to resist gastric proteolysis (Bouzerzour *et al.,* 2012). Maillard glycation of WPI-Lac revealed that these structural changes in native proteins indicate a clear band in 60 mins of gastric digestion (Figure 14); this result is consistent with that of Joubran *et al.* (2017) when protein is glycated with glucose and galactose. These MRPs surprisingly have the potential to enhance the digestibility of proteins compared with the native protein in the digestive tract. Liu and Zhong, (2013) examined the whey protein glycates with reducing saccharides via MR to improve the functional properties of food proteins. By contrast, BLG showed resistance to pepsin hydrolysis in gastric digestion. This result is in accordance with Böttger *et al.* (2013) and Halabi *et al.* (2020). At the end of gastric digestion, a smeared band appeared in glycate and shifted upward by ~25 kDa Figure 14, due to the increase in molecular weight protein, indicating the

conjugation of WPI and lactose had occurred. Undigested BLG in the gastric phase enters the intestine. The low pH will trigger the pancreatic enzymes, such as trypsin and chymotrypsin. Dupont and Mackie (2015) mentioned that the allergenicity of protein can be elicited when the undigested protein in the gastrointestinal tract encounters immune cells whilst passing through the intestinal epithelial barrier. This finding has led to scientific investigations on intestinal proteolysis for the assessment of protein allergenicity; however, the effect of pH on intestinal barriers has not been thoroughly investigated because most researchers focus on gastric pH.

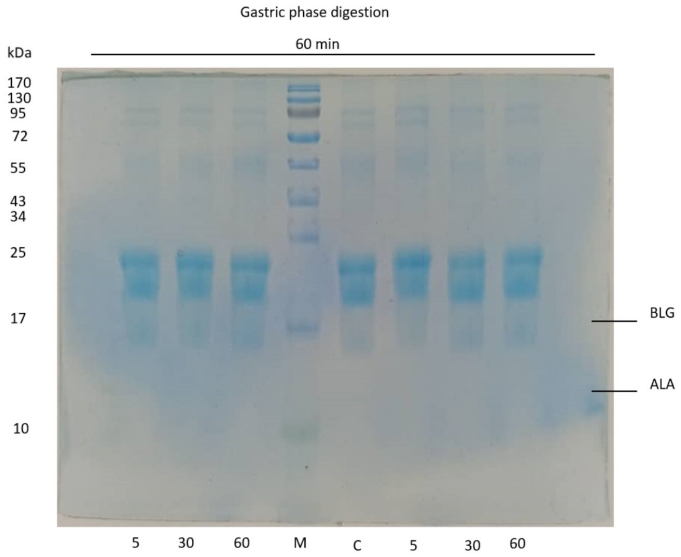

Figure 14. Electrophoresis pattern of WPI-Lac Day 3 during in the in-vitro gastric infant digestion as determined by SDS-PAGE under reducing conditions. At early 30 mins of gastric digestion, the ALA of WPI-Lac partially hydrolyzed and after 60 mins BLG shown significantly to proteolysis in the in-vitro infant gastric digestion using pepsin. C represent WPI as control and M represent protein marker.

4. Conclusion

This study has shown conjugated whey protein with lactose at day 3 promising the extent of conjugation and preventing the formation of advanced MRPs. Light brown colour form and shifted upward band in SDS-PAGE indicated MR has occurred in the sample. Twenty -five percent of free amino acid in proteins were conjugated with lactose at day 3 shown in *ortho*-phthaldehyde data, whereas this conjugate exhibited adequate potency to react with free radicals as shown approximately 30% in ABTS radical scavenging activity. In addition, WPI-lactose at day 3 showed greater solubility which indicated high digestibility in *in-vitro* digestion In the present study, protein glycates formed through MR increased their susceptibility to pepsin hydrolysis when ALA showed a clear staining band at the end of gastric digestion. The resistance of BLG was unaffected by the dry MR with lactose. Future research should quantify the protein glycates on the basis of the calculation reaction rate constant using fluorescence laser densitometry for the kinetics digestion of glycates during gastric digestion. Future work should focus on MRPs with lactose during duodenal digestion in infants. Thus, the effect on protein digestibility in the gastrointestinal tract of infants can be identified.

Conflict of interest

The authors declare no competing interests.

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References

Ahn, J., Cao, M.J., Yu, Y.Q. and Engen, J.R. (2013). Accessing the reproducibility and specificity of pepsin and other aspartic proteases. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics*, 1834(6), 1222-1229. https://doi.org/10.1016/j.bbapap.2012.10.003

Ballard, O. and Morrow, A.L. (2013). Human milk composition: nutrients and bioactive factors. *Pediatric Clinics,* 60(1), 49-74. https://doi.org/10.1016/j.pcl.2012.10.002

Boostani, S., Aminlari, M., Moosavi-Nasab, M., Niakosari, M. and Mesbahi, G. (2017). Fabrication and characterisation of soy protein isolate-grafted dextran biopolymer: A novel ingredient in spray-dried soy beverage formulation. *International Journal of Biological Macromolecules*, 102, 297-307. https://doi.org/10.1016/j.ijbiomac.2017.04.019

Böttger, F.H., Etzel, M.R. and Lucey, J.A. (2013). *In vitro* infant digestion of whey protein–dextran glycates. *Food Digestion*, 4(2-3), 76-84. https://doi.org/10.1007/s13228-013-0032-6

Bouzerzour, K., Morgan, F., Cuinet, I., Bonhomme, C., Jardin, J., Le Huërou-Luron, I. and Dupont, D. (2012). *In vivo* digestion of infant formula in piglets: protein digestion kinetics and release of bioactive peptides. *British Journal of Nutrition*, 108(12), 2105-2114. https://doi.org/10.1017/S000711451200027X

Chatterton, D.E., Nguyen, D.N., Bering, S.B. and Sangild, P.T. (2013). Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *The International Journal of Biochemistry and Cell Biology,* 45(8), 1730-1747. https://doi.org/10.1016/j.biocel.2013.04.028

Chen, W., Ma, X., Wang, W., Lv, R., Guo, M., Ding, T., Ye X., Miao, S. and Liu, D. (2019). Preparation of modified whey protein isolate with gum acacia by ultrasound maillard reaction. *Food Hydrocolloids*, 95, 298-307. https://doi.org/10.1016/j.foodhyd.2018.10.030

Chevalier, F., Chobert, J.M., Popineau, Y., Nicolas, M.G. and Haertlé, T. (2001). Improvement of functional properties of β-lactoglobulin glycated through the Maillard reaction is related to the nature of the sugar. *International Dairy Journal*, 11(3), 145-152. https://doi.org/10.1016/S0958-6946(01)00040-1

Chevalier, F., Chobert, J.M., Dalgalarrondo, M., Choiset, Y. and Haertlé, T. (2002). Maillard glycation of β-lactoglobulin induces conformation changes. *Food/Nahrung*, 46(2), 58-63. https://doi.org/10.1002/1521-3803(20020301)46:2<58::AID-FOOD58>3.0.CO;2-Y

Coles, L.T., Moughan, P.J. and Darragh, A.J. (2005). *In vitro* digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. *Animal Feed Science and Technology*, 123-124(Part 1), 421-444. https://doi.org/10.1016/j.anifeedsci.2005.04.021

Corzo-Martínez, M., Montilla, A., Megías-Pérez, R., Olano, A., Moreno, F.J. and Villamiel, M. (2014). Impact of high-intensity ultrasound on the formation of lactulose and Maillard reaction glycoconjugates. *Food Chemistry,* 157, 186-192. https://doi.org/10.1016/j.foodchem.2014.01.072

Crittenden, R.G. and Bennett, L.E. (2005). Cow's milk allergy: a complex disorder. *Journal of the American College of Nutrition*, 24(Suppl. 6), 582S-591S. https://doi.org/10.1080/07315724.2005.10719507

Damodaran, S. (2007). Amino acids, peptides, and proteins In Damodaran, S., Parkin, K.L. and Fennema, O.R. (Eds.) Fennema's Food Chemistry. 4th ed., p. 217-329. Florida, USA: CRC Press.

Damodaran, S., Parkin, K.L. and Fennema, O.R. (Eds.) Fennema's Food Chemistry. 4th ed. Florida, USA: CRC press.

de Oliveira, F.C., Coimbra, J.S.D.R., E.B., Zuñiga, A.D.G. and Rojas, E.E.G. (2016). Food protein-polysaccharide conjugates obtained via the Maillard reaction: A review. *Critical Reviews in Food Science and Nutrition,* 56(7), 1108-1125. https://doi.org/10.1080/10408398.2012.755669

Deng, Y., Govers, C., Tomassen, M., Hettinga, K. and Wichers, H.J. (2020). Heat treatment of β-lactoglobulin affects its digestion and translocation in the upper digestive tract. *Food Chemistry,* 330, 127184. https://doi.org/10.1016/j.foodchem.2020.127184

Donovan, S.M. (2019). Human Milk: Composition, Clinical Benefits and Future Opportunities. In Donovan, S.M., German, J.B., Lönnerdal, B. and Lucas, A. (Eds.). Human milk proteins: composition and physiological significance. *Nestlé Nutrition Institute Workshop*, 90, 93-101. https://doi.org/10.1159/000490298

Dupont, D. and Mackie, A.R. (2015). Static and dynamic in vitro digestion models to study protein stability in the gastrointestinal tract. *Drug Discovery Today: Disease Models*, 17-18, 23-27. https://doi.org/10.1016/j.ddmod.2016.06.002

Egger, L., Ménard, O., Baumann, C., Duerr, D., Schlegel, P., Stoll, P., Vergères G., Dupont D. and Portmann, R. (2019). Digestion of milk proteins: Comparing static and dynamic in vitro digestion systems with in vivo data. *Food Research International,* 118, 32-39. https://doi.org/10.1016/j.foodres.2017.12.049

Fu, T.J., Abbott, U.R. and Hatzos, C. (2002). Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid a comparative study. *Journal of Agricultural and Food Chemistry,* 50(24), 7154-7160. https://doi.org/10.1021/jf020599h

Gan, J., Bornhorst, G.M., Henrick, B.M. and German, J.B. (2018). Protein digestion of baby foods: study approaches and implications for infant health. *Molecular Nutrition and Food Research,* 62(1), 1700231. https://doi.org/10.1002/mnfr.201700231

Golkar, A., Milani, J.M. and Vasiljevic, T. (2019). Altering allergenicity of cow's milk by food processing for applications in infant formula. Critical reviews in *Food Science and Nutrition*, 59(1), 159-172. https://doi.org/10.1080/10408398.2017.1363156

Halabi, A., Croguennec, T., Bouhallab, S., Dupont, D. and Deglaire, A. (2020). Modification of protein structures by altering the whey protein profile and heat treatment affects *in vitro* static digestion of model infant milk formulas. *Food and Function*, 11(8), 6933-6945. https://doi.org/10.1039/D0FO01362E

Hernández-García, S., Salazar-Montoya, J.A. and Totosaus, A. (2016). Emulsifying properties of food proteins conjugated with glucose or lactose by two methods (Spray-Drying or freeze-drying). *International Journal of Food Properties*, 19(3), 526-536. https://doi.org/10.1080/10942912.2015.1033551

Høst, A. (2002). Frequency of cow's milk allergy in childhood. *Annals of Allergy, Asthma and Immunology*, 89(6), 33-37. https://doi.org/10.1016/S1081-1206(10)62120-5

Hur, S.J., Lim, B.O., Decker, E.A. and McClements, D.J. (2011). *In vitro* human digestion models for food applications. *Food Chemistry,* 125(1), 1-12. https://doi.org/10.1016/j.foodchem.2010.08.036

Huang, X., Tu, Z., Xiao, H., Wang, H., Zhang, L., Hu, Y., Zhang, Q. and Niu, P. (2012). Characteristics and antioxidant activities of ovalbumin glycated with different saccharides under heat moisture treatment. *Food Research International*, 48(2), 866–872. https://doi.org/10.1016/j.foodres.2012.06.036

Jiang, Z. and Brodkorb, A. (2012). Structure and antioxidant activity of Maillard reaction products from α-lactalbumin and β-lactoglobulin with ribose in an aqueous model system. *Food Chemistry,* 133 (3), 960-968. https://doi.org/10.1016/j.foodchem.2012.02.016

Joubran, Y., Moscovici, A., Portmann, R. and Lesmes, U. (2017). Implications of the Maillard reaction on bovine alpha-lactalbumin and its proteolysis during in vitro infant digestion. *Food and Function,* 8(6), 2295-2308. https://doi.org/10.1039/C7FO00588A

Julmohammad, N. (2017). Synthesis of Lactose Phosphate and its Conjugation with Whey Proteins. New Zealand: Semantic Scholar Publishing.

Kato, Y., Matsuda, T., Kato, N. and Nakamura, R. (1988). Browning and protein polymerization induced by amino carbonyl reaction of ovalbumin with glucose and lactose. *Journal of Agricultural and Food Chemistry,* 36(4), 806–809. https://doi.org/10.1021/jf00082a034

Kato, A., Mifuru, R., Matsudomi, N. and Kobayashi, K. (1992). Functional Casein-Poly saccharide Conjugates Prepared by Controlled Dry Heating. *Bioscience, Biotechnology, and Biochemistry*, 56(4), 567–571. https://doi.org/10.1271/bbb.56.567

Kato, Y., Aoki, T., Kato, N., Nakamura, R. and Matsuda, T. (1995). Modification of Ovalbumin with Glucose 6-Phosphate by Amino–Carbonyl Reaction. Improvement of Protein Heat Stability and Emulsifying Activity. *Journal of Agricultural and Food Chemistry*, 43(2), 301–305. https://doi.org/10.1021/jf00050a007

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227(5259), 680-685. https://doi.org/10.1038/227680a0

Li, Z., Luo, Y. and Feng, L. (2011). Effects of Maillard reaction conditions on the antigenicity of α-lactalbumin and β-lactoglobulin in whey protein conjugated with maltose. *European Food Research and Technology*, 233(3), 387–394. https://doi.org/10.1007/s00217-011-1532-7

Liu, G. and Zhong, Q. (2013). Thermal aggregation properties of whey protein glycated with various saccharides. *Food Hydrocolloids,* 32(1), 87-96. https://doi.org/10.1016/j.foodhyd.2012.12.008

Liu, L., Li, X., Du, L., Zhang, X., Yang, W. and Zhang, H. (2019). Effect of ultrasound assisted heating on structure and antioxidant activity of whey protein peptide grafted with galactose. *Lebensmittel-Wissenschaft and Technologie*, 109, 130-136. https://doi.org/10.1016/j.lwt.2019.04.015

Liu, Q., Kong, B., Han, J., Sun, C. and Li, P. (2014). Structure and antioxidant activity of whey protein isolate conjugated with glucose via the Maillard reaction under dry-heating conditions. *Food Structure,* 1(2), 145-154. https://doi.org/10.1016/j.foostr.2013.11.004

Lönnerdal, B. (2003). Nutritional and physiologic significance of human milk proteins. *The American Journal of Clinical Nutrition,* 77(6), 1537S-1543S. https://doi.org/10.1093/ajcn/77.6.1537S

Lucas-González, R., Viuda-Martos, M., Pérez-Alvarez, J.A. and Fernández-López, J. (2018). *In vitro* digestion models suitable for foods: Opportunities for new fields of application and challenges. *Food Research International*, 107, 423-436. https://doi.org/10.1016/j.foodres.2018.02.055

Maathuis, A., Havenaar, R., He, T. and Bellmann, S. (2017). Protein digestion and quality of goat and cow milk infant formula and human milk under simulated infant conditions. *Journal of Pediatric Gastroenterology and Nutrition*, 65(6), 661. https://doi.org/10.1097/MPG.0000000000001740

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D. and Picque, D. (2014). Validation of a new *in vitro* dynamic system to simulate infant digestion. *Food Chemistry*, 145, 1039-1045. https://doi.org/10.1016/j.foodchem.2013.09.036

Ménard, O., Bourlieu, C., De Oliveira, S.C., Dellarosa, N., Laghi, L., Carrière, F. and Deglaire, A. (2018). A first step towards a consensus static *in vitro* model for simulating full-term infant digestion. *Food Chemistry*, 240, 338-345. https://doi.org/10.1016/j.foodchem.2017.07.145

Meyer, R., Groetch, M. and Venter, C. (2018). When should infants with cow's milk protein allergy use an amino acid formula? A practical guide. *The Journal of Allergy and Clinical Immunology: In Practice,* 6

(2), 383-399. https://doi.org/10.1016/j.jaip.2017.09.003

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T.., Bourlieu, C., Carriere, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Menard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W. and Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food–an international consensus. *Food and Function*, 5(6), 1113-1124. https://doi.org/10.1039/C3FO60702J

Moimaz, S.A.S., Amaral, M.A., Garbin, C.A.S., Saliba, T.A. and Saliba, O. (2018). Caries in children with lactose intolerance and cow's milk protein allergy. *Brazilian Oral Research*, 32, 0091. https://doi.org/10.1590/1807-3107bor-2018.vol32.0091

Morales, F.J. and Van Boekel, M.A.J.S. (1998). A study on advanced Maillard reaction in heated casein/sugar solutions: colour formation. *International Dairy Journal*, 8(10-11), 907-915. https://doi.org/10.1016/S0958-6946(99)00014-X

Mulet-Cabero, A.I., Mackie, A.R., Wilde, P.J., Fenelon, M.A. and Brodkorb, A. (2019). Structural mechanism and kinetics of *in vitro* gastric digestion are affected by process-induced changes in bovine milk. *Food Hydrocolloids*, 86, 172-183. https://doi.org/10.1016/j.foodhyd.2018.03.035

Nooshkam, M. and Madadlou, A. (2016). Microwave-assisted isomerisation of lactose to lactulose and Maillard conjugation of lactulose and lactose with whey proteins and peptides. *Food Chemistry*, 200, 1-9. https://doi.org/10.1016/j.foodchem.2015.12.094

Nooshkam, M., Varidi, M. and Bashash, M. (2019). The Maillard reaction products as food-born antioxidant and anti-browning agents in model and real food systems. *Food Chemistry*, 275, 644-660. https://doi.org/10.1016/j.foodchem.2018.09.083

Santos, I.J.B., Hernandez, H.L.H., Costa, M.H.C., Júnior, J.A.D.Q.L. and dos Reis Coimbra, J.S. (2019). Conjugates of α-lactalbumin, β-lactoglobulin, and lysozyme with polysaccharides: Characterization and techno-functional properties. *Food Research International*, 116, 492-498. https://doi.org/10.1016/j.foodres.2018.08.065

Pekar, J., Ret D. and Untersmayr E. (2018). Stability of allergens. *Molecular Immunology*, 100, 14-20. https://doi.org/10.1016/j.molimm.2018.03.017

Picariello, G., Mamone, G., Nitride, C., Addeo, F. and Ferranti, P. (2013). Protein digestomics: Integrated platforms to study food-protein digestion and derived functional and active peptides. *Trends in Analytical Chemistry*, 52, 120–134. https://doi.org/10.1016/j.trac.2013.08.001

Shani-Levi, C., Alvito, P., Andrés, A., Assunção, R., Barberá, R., Blanquet-Diot, S., Bourlieu, C., Brodkorb, A., Cilla, A., Deglaire, A., Denis, S., Dupont, D., Heredia, A., Karakaya, S., Giosafatto, C.V.L., Mariniello, L., Martins, C., Ménard, O., El, S.N., Elizabeth Vegarud G., Ulleberg E. and Lesmes, U. (2017). Extending *in vitro* digestion models to specific human populations: Perspectives, practical tools and bio-relevant information. *Trends in Food Science and Technology*, 60, 52–63. https://doi.org/10.1016/j.tifs.2016.10.017

Su, J.F., Huang, Z., Yuan, X.Y., Wang, X.Y. and Li, M. (2010). Structure and properties of carboxymethyl cellulose/soy protein isolate blend edible films crosslinked by Maillard reactions. *Carbohydrate Polymers*, 79(1), 145-153. https://doi.org/10.1016/j.carbpol.2009.07.035

Nguyen, T.P.T. (2017). Digestibility and structural changes of ingredients in infant formulae during the gastrointestinal digestion. Australia: The University of Queensland, PhD. Dissertation.

Nguyen, T.P.T., Bhandari, B., Cichero, J. and Prakash, S. (2015). A comprehensive review on in vitro digestion of infant formula. *Food Research International,* 76(3), 373-386. https://doi.org/10.1016/j.foodres.2015.07.016

Teodorowicz, M., Van Neerven, J. and Savelkoul, H. (2017). Food processing: The influence of the maillard reaction on immunogenicity and allergenicity of food proteins. *Nutrients*, 9(8), 835. https://doi.org/10.3390/nu9080835

Tu, Z.C., Zhang, L., Wang, H., Huang, X.Q., Zhang, L. and Yang, K. (2014). Structure and antioxidant activity of milk model systems after microwave heating. *Food Science and Technology Research,* 20 (2), 345-355. https://doi.org/10.3136/fstr.20.345

Verhoeckx, K., Bøgh, K.L., Dupont, D., Egger, L., Gadermaier, G., Larré, C., Mackie, A., Menard, O., Adel-Patient, K., Picariello, G., Portmann, R., Smit, J., Turner, P., Untersmayr, E. and Epstein, M.M. (2019). The relevance of a digestibility evaluation in the allergenicity risk assessment of novel proteins. Opinion of a joint initiative of COST action ImpARAS and COST action INFOGEST. *Food and Chemical Toxicology*, 129, 405-423. https://doi.org/10.1016/j.fct.2019.04.052

Wada, Y. and Lönnerdal, B. (2014). Effects of different industrial heating processes of milk on site-specific protein modifications and their relationship to *in vitro* and *in vivo* digestibility. *Journal of*

Agricultural and Food Chemistry, 62(18), 4175-4185. https://doi.org/10.1021/jf501617s

Wal, J.M. (2004). Bovine milk allergenicity. *Annals of Allergy, Asthma and Immunology*, 93(5), S2-S11. https://doi.org/10.1016/S1081-1206(10)61726-7

Wefers, D., Bindereif, B., Karbstein, H.P. and Van Der Schaaf, U.S. (2018). Whey protein-pectin conjugates: Linking the improved emulsifying properties to molecular and physico-chemical characteristics. *Food Hydrocolloids*, 85, 257-266. https://doi.org/10.1016/j.foodhyd.2018.06.030

Ye, A., Cui, J., Carpenter, E., Prosser, C. and Singh, H. (2019). Dynamic *in vitro* gastric digestion of infant formulae made with goat milk and cow milk: Influence of protein composition. *International Dairy Journal*, 97, 76-85. https://doi.org/10.1016/j.idairyj.2019.06.002

Yu, J.J., Ji, H., Chen, Y., Zhang, Y.F., Zheng, X.C., Li, S.H. and Chen, Y. (2020). Analysis of the glycosylation products of peanut protein and lactose by cold plasma treatment: Solubility and structural characteristics. *International Journal of Biological Macromolecules*, 158, 1194-1203. https://doi.org/10.1016/j.ijbiomac.2020.04.255

Zhang, Q., Li, L., Lan, Q., Li, M., Wu, D., Chen, H., Yaowen, L., Derong, L., Wen, Q., Zhiqing, Z., Jiang, J. and Yang, W. (2019). Protein glycosylation: A promising way to modify the functional properties and extend the application in food system. *Critical reviews in Food Science and Nutrition*, 59(15), 2506-2533. https://doi.org/10.1080/10408398.2018.1507995

Zhang, X., Li, X., Liu, L., Wang, L., Bora, A.F.M. and Du, L. (2020). Covalent conjugation of whey protein isolate hydrolysates and galactose through Maillard reaction to improve the functional properties and antioxidant activity. *International Dairy Journal*, 102, 104584. https://doi.org/10.1016/j.idairyj.2019.104584

Zhong, J.Z., Xu, Y.J., Liu, W., Liu, C.M., Luo, S.J. and Tu, Z.C. (2013). Antigenicity and functional properties of β-lactoglobulin conjugated with fructo-oligosaccharides in relation to conformational changes. *Journal of Dairy Science,* 96(5), 2808-2815. https://doi.org/10.3168/jds.2012-6259

Zhu, D., Damodaran, S. and Lucey, J.A. (2008). Formation of whey protein isolate (WPI)−dextran conjugates in aqueous solutions. *Journal of Agricultural and Food Chemistry*, 56(16), 7113-7118. https://doi.org/10.1021/jf800909w

