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Heat treatment of β-lactoglobulin affects its digestion and translocation in the upper digestive tract

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ABSTRACT

Heat treatment is a commonly applied unit operation in the processing of β-lactoglobulin containing products. This does, however, influence its structure and thereby impacts its activity and digestibility. We describe how various heat-treatments of β-lactoglobulin change the digestibility using a modified version of the current consensus INFOGEST protocol. Additionally, protein was investigated for its translocation over the intestinal epithelial barrier, which would bring them in contact with immune cells. The extent of gastric digestibility was higher when the protein structure was more modified, while the influence of glycation with lactose was limited. Translocation studies of protein across Caco-2 cell monolayers showed a lower translocation rate of protein heated in solution compared to the others. Our study indicates that structural modifications after different heat-treatments of β-lactoglobulin increase in particular gastric digestibility and the translocation efficiency across intestinal epithelial cells.

1. Introduction

Cow's milk allergy is one of the major food allergies, especially in children. The prevalence of cow's milk allergy is estimated to be 2.5% amongst children in the Western Hemisphere (Sicherer & Sampson, 2010). There is some controversy, based on methodological issues, as to whether the prevalence is increasing (Flom & Sicherer, 2019). Cow's milk contains about 3.5% protein in which at least 25 different proteins were identified that may act as an allergen. β-Lactoglobulin (BLG), which accounts for approximately one tenth of all proteins in cow's milk, is one of the major allergens (Savilahti & Kuitunen, 1992).

Thermal processing of milk is an essential step in the dairy industry to prolong its shelf life. However, these heat treatments may lead to unfolding, aggregation, and glycation with lactose (Morr, 1985). In our study, native BLG was heated in the presence or absence of lactose following 3 different methods: wet-heating at 60 °C for 72 h, low-temperature dry-heating at water activity 0.65, 50 °C for 9 h and high-temperature dry-heating at water activity 0.59, 130 °C for 10 min. Wet-heating increased the molecular weight and hydrophobicity of BLG significantly, regardless of the presence of lactose. High- or low-temperature dry-heating led to advanced or early stage glycation of BLG, respectively, in the presence of lactose, but less significant structural changes of BLG were observed in the soluble fraction, as reported earlier (Deng et al., 2019). These structure modifications can either reduce or enhance the allergenic potential of BLG depending on the processing conditions (Rahaman, Vasiljevic, & Ramchandran, 2016). Glycation is linked to masking of epitopes and may result in reduced protein binding by IgE in cow's milk allergic patients (Taheri-Kafrani et al., 2009). Antigenicity of BLG was reported to increase with increasing heating temperature until 90 °C, but to decrease at even higher temperatures (Bu, Luo, Zheng, & Zheng, 2009). Moreover, thermal-processing-induced structure modifications may influence the digestibility of the protein, which could further alter its immunogenicity, as the sensitizing capacity of BLG was observed to be decreased along the digestion process (Bogh, Barkholt, & Madsen, 2013).

Like many other food allergens, BLG resists gastric digestion, mainly due to its stability at the acidic pH of the gastric environment and its compact structure. This may result in increased exposure of larger fragments to the gut-associated lymphoid tissue, possibly contributing to the development of an allergic reaction (Moreno, 2007). The consensus *in vitro* digestion protocol (INFOGEST) confirmed the resistance of BLG to gastric digestion. Using this protocol, BLG was reported to be mainly digested in the intestinal phase, again reflecting physiological processes (Egger, Menard, Delgado-Andrade, Alvito, Assuncao, Balance, et al., 2016). Prolonged survival of BLG was also observed in the jejunum in an *in vivo* study (Sanchon, Fernandez-Tome, Miralles,

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Hernandez-Ledesma, Tome, Gaudichon, et al., 2018). In addition to support digestion, the gastro-intestinal (GI) tract hosts a large population of immune cells and lymphoid tissue as it is an important route of pathogenic entry. It is thus essential to study the interaction between food proteins like BLG and the immune system based on a clear understanding of its digestibility, which can be influenced by the type and severity of thermal processing that has been applied. For instance, potential cleavage sites for digestive enzymes buried in the native form of a protein might be exposed due to unfolding. BLG heated at around 70 °C in solution becomes susceptible to gastric digestion (Macierzanka et al., 2012). Contrastingly, cleavage sites could be modified because of glycation and crosslinking, which can both impair the digestibility of BLG (Corzo-Martinez, Soria, Belloque, Villamiel, & Moreno, 2010; Pinto et al., 2014).

Allergens may be sampled from the intestinal luminal content by antigen presenting cells (Farache et al., 2013). In addition, allergens may also actively pass through the intestinal epithelial layer and directly contact with a range of immune cells (Hepworth et al., 2013; Price, Ackland, & Suphioglu, 2013). Such active transport through the epithelial layer may be studied with Caco-2 cells that are differentiated into small intestinal-like enterocytes, which have been used widely for intestinal translocation studies (Bodinier et al., 2007). Literature on the impact of thermal processing of BLG on its digestibility and intestinal epithelial passage is rather scattered, which was the reason for us to analyse these effects in one study. Such information can contribute to better evaluation of the impact of BLG processing on the intestinal immunological response.

2. Material and methods

2.1. Chemicals

All chemicals were purchased from Sigma Aldrich (St Louis, Missouri, USA) unless otherwise stated.

2.2. Sample preparation

β-Lactoglobulin (BLG) in its native form was isolated from raw cow's milk as published (de Jongh, Groneveld, & de Groot, 2001), heat-treated, centrifuged, dialyzed and protein concentration measured as described previously (Deng et al., 2019). Briefly, BLG was heated in solution at 60 °C for 72 h for the wet-heating (W) samples, low-temperature dry-heating (L; a_w 0.65, 50 °C for 9 h) and high-temperature dry-heating (H; a_w 0.53, 130 °C for 10 min) in the presence or absence of lactose.

2.3. In vitro gastrointestinal digestion

The applied *in vitro* digestion protocol is a scaled-down adaptation of the INFOGEST model (Alegria et al., 2015). BLG samples were diluted to 2 mg/mL with 140 mM NaCl + 5 mM KCl solution to mimic the oral situation after which we moved forward with 200 μL. For mimicking gastric digestion, the pH was set to 2 with 1 M HCl and 6.67 μL of pepsin solution (1092 U/mL dissolved in 0.1 M HCl) was added. Samples were incubated for 1 h while gently shaking at 37 °C. After pH was set to 5.8 with 1 M $NaHCO_3$, 1.6 μg pancreatin (6.84 U/mg trypsin activity) and 2.36 μg α-chymotrypsin (65.61 U/mg) from porcine pancreas, and bile salts (453.8 μg sodium taurocholate and 415.0 μg sodium glycodeoxycholate) dissolved together in 15 μL of 0.1 M $NaHCO_3$ were added. Again, the pH of samples was adjusted to 6.5 by 1 M $NaHCO_3$ after which the samples were incubated at 37 °C while gently shaking to mimic the intestinal digestion. As a control we prepared a digest which contained all volumes, pH changes and enzymes but without addition of BLG.

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Non-reducing SDS-PAGE was performed by mixing 12 μg of protein sample with a corresponding volume of NuPAGE® LDS Sample Buffer (4X) (ThermoFischer, Waltham, Massachusetts, USA), followed by boiling for 5 min and loading on 10% Bis-Tris gels (ThermoFischer, Waltham, Massachusetts, USA) together with a pre-stained Precision Plus Protein™ Dual Xtra marker (Bio-Rad, Hercules, California, USA). Gels were running under 160 V for 50 min, stained by PageBlue™ Protein Staining Solution (ThermoFischer, Waltham, Massachusetts, USA) and de-stained by MilliQ water and scanned by Universal Hood III using Image Lab version 4.1 software (Bio-Rad, Hercules, California, USA). With the same software, the lanes of the gels were analysed and the percentage of undigested protein was calculated based on the band density ratio of original loading BLG samples.

2.5. Ortho-phthalaldehyde (OPA) measurement

Protein samples' free amino groups along digestion were measured by OPA as previously described (Deng et al., 2019). Degree of hydrolysis (DH, %) for gastric or intestinal digestion was calculated separately by the following equation:

$$DH\,(\%) = \left(\frac{\Delta \#NH_{2digestion}}{\#peptide\ bonds\ of\ BLG}\right) \times 100\%$$

where $\#NH_2$ is the number of free amino groups per protein molecule as calculated by using the concentration of free amino groups divided by the concentration of protein, Δ is the difference of number before and after digestion.

2.6. ANS measurement

To determine the change of surface hydrophobicity during digestion, ANS measurement was applied like previously described (Deng et al., 2019).

2.7. Fluorescent labelling of protein

Fluorescein isothiocyanate (FITC) isomer I was used to label and track the translocation of protein through a Caco-2 monolayer. Briefly, 1 mg/mL FITC dissolved in DMSO was added with a volume ratio 1:10 to protein samples in borate buffer (10 mM, pH 9), resulting in a final protein concentration of 1 mg/mL and incubated overnight at 4 °C. The unbound FITC was filtered out by passing the mixture through a NAP-10 column (GE healthcare, Chicago, Illinois, USA). The FITC labelled protein sample was eluted using 0.1 M PBS (pH 7.4) at a final protein concentration of 500 μg/mL.

2.8. Translocation of protein through the Caco-2 monolayer

Caco-2 cells (ATCC, Manassas, Virginia, USA) between passage number 30 and 40 were grown in a 6 or 24-well insert transwell plate (Greiner bio-one, Alphen a/d Rijn, The Netherlands) using Dulbecco's Modified Eagles Medium (DMEM) with 4.5 g/L glucose, 4 mM L-glutamine and 25 mM HEPES (Gibco, Thermo Fisher, Waltham, Massachusetts, USA), supplemented with 10% heat inactivated fetal bovine serum (Invitrogen, Paisley, UK). Cells were seeded at a concentration of 0.225×10^6 cells/mL. A volume of 150 μL for 24-well and 2 mL for 6-well was added to the apical side, and 750 μL or 4 mL medium was added to the basolateral side of the transwells, respectively. The cells developed into small intestinal like cells after 21 days of incubation with medium being changed three times a week.

To study BLG translocation, the transepithelial electrical resistance (TEER) value of the Caco-2 monolayers was measured (shown in Fig.

S1) and only monolayers with a TEER value higher than 600 Ω*cm^2 were used. The apical medium of both 6 and 24-well inserts was replaced by FITC labelled protein diluted 2 times (final protein concentration 250 mg/mL) using DMEM medium without phenol red (Gibco, Thermo Fisher, Waltham, Massachusetts, USA) or medium only as control. After 6 or 24 h of incubation, basolateral fractions were collected. Fluorescence intensity of the basolateral medium from 24-well inserts was recorded by Infinite® 200 PRO NanoQuant with i-control software (Tecan, Männedorf, Switzerland) in arbitrary units using 495 nm and 525 nm as excitation and emission wavelength. The final protein concentration was determined using a standard curve of each individual sample with a concentration range of 0–500 μg/mL. The basolateral medium from 6-well inserts was concentrated using Amicon Ultra-0.5 mL Centrifugal Filters (Merck Millipore, Massachusetts, USA) with a cut-off of 3 kDa from 4 mL to 48 μL. From these concentrates, 9 μL was used for SDS-PAGE as described above and the gel was scanned by Universal Hood III using Image Lab version 4.1 software (Bio-Rad, Hercules, California, USA) with FITC detection settings.

2.9. Statistics

The statistical analysis including Dunnett's Test, T test and correlation analysis was performed using Prism 6 software with $p < 0.05$ considered to be significant. The PCA plot was generated with the dataset of variables being mean centred and weighted by 1/standard deviation by Unscrambler software (CAMO, Oslo, Norway).

3. Results

3.1. Wet-heated BLG was digested much faster in vitro than native or high- or low-temperature dry-heated BLG

Following heat treatment in the presence or absence of lactose, we observed a minimal effect of low-temperature dry-heating on the polymerisation degree of BLG and the strongest effect of wet-heating, independent of the presence of lactose (Fig. 1). An intermediate effect on polymerisation of high-temperature dry-heating was observed, but unlike for the other treatments, polymerisation of BLG was enhanced by the presence of lactose. After heating, we digested the BLG samples in a two-step (i.e. gastric and intestinal) *in vitro* digestion model. SDS-PAGE revealed that the monomeric (18 kDa) and dimeric (36 kDa) bands of native BLG remained intact after the gastric phase. Similar to native BLG, the gastric digestion of low-temperature dry-heated BLG was very limited. High-temperature dry-heated BLG appeared to be digested somewhat more than both these samples in the gastric phase. Contrastingly, a considerably larger proportion of wet-heated BLG appeared to be digested in the gastric phase, as large aggregates were no longer visible. For none of the heat treatments, the presence of lactose appeared to affect the degradation of BLG during the gastric phase.

A quantitative analysis of the observed protein bands using density scanning revealed that for native BLG approximately 96% of protein remained undigested following gastric digestion (Table 1). Despite that high- and low-temperature dry-heated samples demonstrated a similar digestion pattern compared to native BLG as described above, these treatments did result in a significant higher gastric digestion, with undigested protein in these samples ranging between 63 and 75%. Wet-heated BLG samples were digested most extensively in the gastric phase, with only about 38% undigested protein remaining detectable.

During the intestinal digestion, the samples analysed after 10 min demonstrated that undigested protein, albeit already at a lower intensity, was still clearly visible (Fig. 1). Surprisingly, digestion of BLG that was dry-heated in the absence of lactose resulted in the presence of BLG fractions smaller than the monomeric BLG. After 120 min of intestinal digestion, native BLG was almost completely degraded with only a faint monomeric BLG band remaining. We observed a similar impact of intestinal digestion for all the other samples. The remaining band of undigested BLG was the clearest for the low-temperature dry-heated BLG in the presence of lactose.

Quantitative analysis confirmed the presence of undigested BLG in samples after ten minutes of intestinal digestion (Table 1). Although a clearly reduced amount of undigested protein was observed in all samples, this was most pronounced for wet-heated BLG of which only 6 and 15% was still detectable in the absence and presence of lactose, respectively. After completing the digestion protocol, all BLG samples were almost completely digested, independent of treatment, as the percentage of remaining undigested protein for all samples was below 10.5% and only the low temperature dry heated BLG was higher compared to native BLG.

3.2. Relationship between gastric digestibility and physicochemical properties of BLG samples

The physicochemical properties of the heat-treated BLG samples have been investigated previously (Deng et al., 2019). Here, we analysed whether there is a relation between these properties and the observed differences in gastric digestibility for these samples. A PCA plot (Fig. 2) and a correlation analysis (Fig. S2) of the physicochemical properties of the variously processed BLG samples versus the gastric digestibility (percentage of digested protein) showed that the proportion of polymers, exposure of hydrophobic regions and percentage of α-helix were positively related to gastric digestibility, as opposed to β-sheet structure and the proportion of monomers that were negatively related.

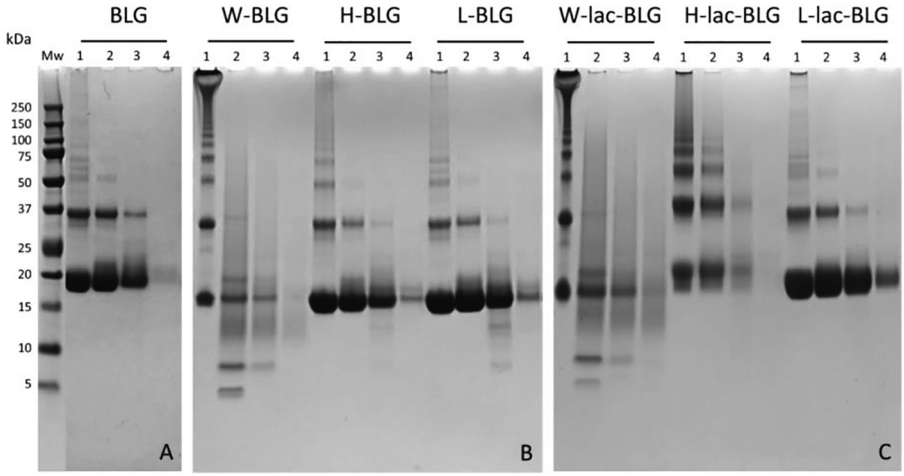

Fig. 1. Wet-heating strongly increased gastric digestion of native BLG. BLG was untreated (native: BLG) or in the absence or presence of lactose wet-heated (W-BLG or W-lac-BLG, respectively), high-temperature dry-heated (H-BLG or H-lac-BLG, respectively) or low-temperature dry-heated (L-BLG or L-lac-BLG, respectively) and subsequently *in vitro* digested according to a modified INFOGEST protocol. Native BLG (A), wet-heated, high- and low-temperature dry-heated BLG in the absence (B) or presence of lactose (C) were loaded on a non-reducing SDS-PAGE (a representative picture was depicted of n = 3 independent digestions) before digestion (lane 1), following gastric digestion (lane 2), following 10 min of intestinal digestion (lane 3) and following 120 min of intestinal digestion (lane 5); Mw: molecular weight marker ranging from 5 to 250 kDa.

Table 1
Percentage of BLG detectable with SDS-PAGE during digestion.

Digestion Phase	BLG	W-BLG	H-BLG	L-BLG	W-lac-BLG	H-lac-BLG	L-lac-BLG
60 min gastric	95.9 ± 3.1	38.7 ± 5.8***	63.5 ± 3.3***	74.6 ± 7.0**	38.1 ± 8.5***	62.7 ± 6.5***	66.9 ± 0.4***
10 min intestinal	77.0 ± 2.0	5.7 ± 2.8***	36.0 ± 4.9***	58.8 ± 6.4**	15.2 ± 1.8***	19.8 ± 3.1***	56.3 ± 3.1***
120 min intestinal	4.5 ± 2.6	0.4 ± 0.5	5.4 ± 0.6	6.7 ± 0.6	3.0 ± 2.3	2.8 ± 2.0	10.5 ± 2.5*

Note: BLG was heat-treated, digested and loaded on a non-reducing SDS-PAGE as described in the legend of Fig. 1. The total amount of protein bands for each sample and time point were quantified using Image Lab software, compared to intensities from non-digested BLG samples and represented as percentages. The values represent averages ± standard deviation of n = 3 independent experiments. Each heat-treated BLG sample was compared to native BLG at each time point and statistical significant differences were calculated with Dunnett's Test. *p < 0.05; **p < 0.01; ***p < 0.001.

3.3. Wet-heated BLG samples were more extensively hydrolysed during gastric digestion compared to other samples

The degree of protein hydrolysis during digestion can be quantified by the percentage of released free amino groups relative to the total number of peptide bonds (161 for BLG). As shown in Fig. 3, the degree of hydrolysis (DH) in the gastric phase for native and dry-heated BLG samples was lower (i.e. around 1%) than for wet-heated BLG samples. For wet-heated BLG, the presence of lactose seemed to increase the DH to a value close to 5% which is significantly higher than for the native and dry-heated BLG samples. Hydrolysis of the native and dry-heated BLG samples occurred mainly during the intestinal phase, with the DH being over 6%. Interestingly, the presence of lactose in the heating process seemed to exhibit an inhibitory effect during the intestinal digestion phase, especially for wet-heated samples, leading to a significantly lower DH value.

3.4. During gastric digestion, hydrophobic regions in wet-heated BLG samples processed in the presence of lactose were lost

Because of the importance of hydrophobicity as a marker for a protein's structural integrity and its importance in uptake of processed BLG by THP-1 macrophages (Deng et al., 2019), changes in hydrophobicity of the samples were measured during digestion. For all samples, the ANS signal decreased during digestion (Fig. 4). Before digestion, the ANS signal for wet-heated BLG samples was significantly higher than for the other samples. The ANS signal of wet-heated BLG in the absence of lactose remained significantly higher compared to native BLG until 10 min of intestinal digestion and remained numerically, but not significantly, higher after completing the intestinal digestion. On the contrary, we observed a strong decrease in hydrophobicity of wet-heated BLG in the presence of lactose during the gastric phase to a value close to native and dry-heated BLG samples, reaching the same level after the entire digestion process.

3.5. Wet-heated BLG was less likely to pass across a Caco-2 epithelial monolayer

More than half of the native BLG and dry-heated BLG samples, and a considerable proportion of wet-heated BLG (> 38%), remained undigested after the gastric digestion (Table 1). Hence, part of the BLG in the samples will enter the small intestine in its undigested form. To understand the passage of undigested protein across epithelial cells, we tested for translocation of BLG across Caco-2 monolayers. To this end, we FITC-labelled BLG samples and applied them for 6 or 24 h to 3 weeks old transwell-grown small intestinal-like Caco-2 cells. The amount of protein transferred from the apical to the basolateral side was determined by measuring the fluorescent signal of the basolateral fraction (Fig. 5A). Slightly < 0.8% of total input fluorescence signal of native BLG was detectable at the basolateral side after 6 h of incubation. Translocation of wet-heated BLG in presence or absence of lactose to the basolateral side was significantly less compared to native BLG (*ca.* 0.1 and 0.3%, respectively). All other samples showed translocation across the Caco-2 monolayer similar to native BLG.

To identify the protein sizes that translocated to the basolateral compartments, we performed SDS-PAGE with concentrated samples. Due to the limited effect from the absence of lactose, we focused on the concentrated basolateral fractions for native BLG and lactose-containing samples only (Fig. 5B). SDS-PAGE analysis showed that the translocated FITC signal mainly corresponds to monomeric BLG in all samples. For the samples with a relative high translocation rate (Fig. 5A) and strong monomeric BLG signal (Fig. 5B left), i.e. native and low-temperature dry-heated BLG in the presence of lactose, there were clear fluorescent bands detectable in basolateral medium after 6 h of incubation (Fig. 5B right). Moreover, the intensity of the protein fluorescent bands increased for all samples upon longer incubation time

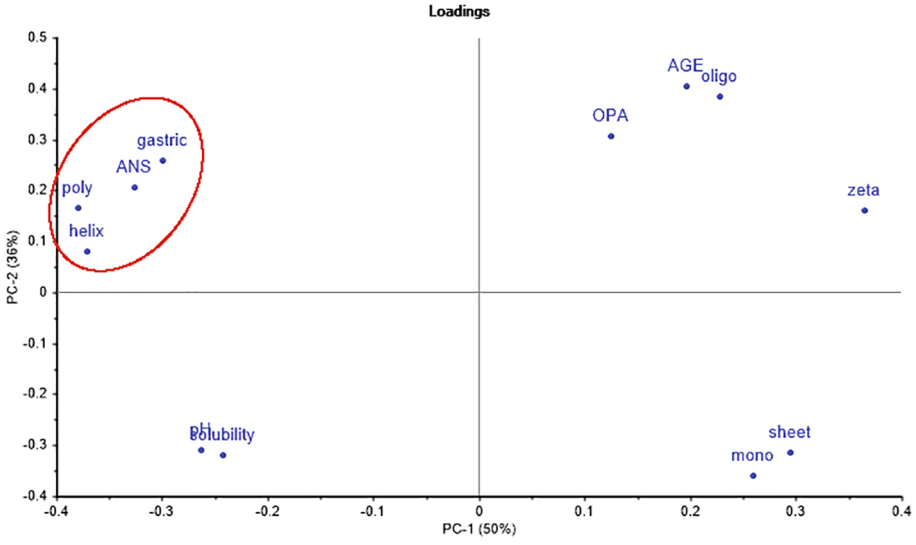

Fig. 2. Gastric digestibility of BLG samples was related to sample hydrophobicity, proportion of polymers and percentage of α-helix. BLG was treated and digested as described in the legend of Fig. 1. The samples were previously analysed for their solubility, pH, AGE formation (AGE), glycation (OPA), percentage of α-helix (helix) or β-sheet (sheet), proportion of monomer and dimer (mono), oligomers (oligo), polymers (poly), exposure of hydrophobic regions (ANS) and surface charge (zeta), and relationships with degree of digestion in the gastric phase (gastric) were investigated using principal component analysis of n = 3–4 independent analysis of each characteristic with components covering 86% of the data. The red oval indicates the clustering of gastric phase digestion with physicochemical characteristics. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

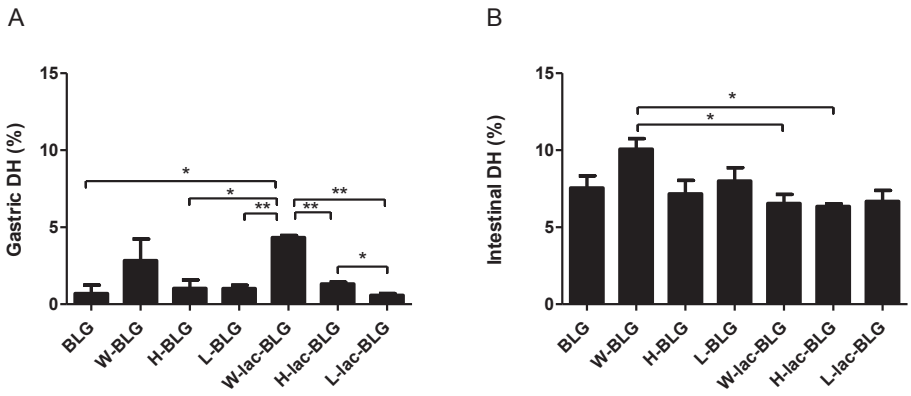

Fig. 3. Digestion of BLG was enhanced by wet-heating but inhibited by the presence of lactose in intestinal phase. BLG was treated as described in the legend of Fig. 1 and the degree of hydrolysis was determined for each sample following gastric (A) and intestinal (B) digestion using OPA analysis. Bars represent averages ± standard deviation of n = 2 independent assays. Statistically significant differences were calculated with a two-tailed unpaired T test with: *p < 0.05; **p < 0.01.

with the Caco-2 cells, indicating continued translocation.

4. Discussion

The native structure of BLG is made up of 9 anti-parallel β-sheets and 1 α-helix (Hamada, Segawa, & Goto, 1996), making it rather compact and resistant to pepsinolysis in the gastric phase and partially resistant to degradation by trypsin in the duodenal phase (Dupont, Mandalari, Molle, Jardin, Leonil, Faulks, et al., 2010). Although several pepsin cleavage sites have been predicted in the amino acid sequence of BLG, the integrity of the β-barrel structure under acid conditions (Mahe, Messing, Thuillier, & Tome, 1991) protects the protein from hydrolysis by rendering the potential cleavage sites inaccessible to this enzyme (Guo, Fox, Flynn, & Kindstedt, 1995). Literature reporting that native BLG is hardly digested in the gastric compartment but mainly in the intestine (Egger et al., 2016) are in line with our findings (Fig. 1 and Table 1), thereby validating the applied *in vitro* digestion protocol. Building on this, we studied a range of well-defined BLG processing conditions, to get a better understanding of the relation between the changes to BLG induced by processing and its digestibility.

Heating BLG in a watery solution has previously been observed to accelerate pepsin digestion (Kitabatake & Kinekawa, 1998; Peram, Loveday, Ye, & Singh, 2013). BLG heated under this condition was reported to exhibit increased flexibility of its tertiary structure and conformational changes (Seo et al., 2010), as well as unfolding of the protein, leading to the formation of aggregates (Peixoto, Trivelli, Andre, Moreau, & Delaplace, 2019). With these structural changes, higher exposure of hydrophobic structures was observed by us (Fig. 3) and also others (Creamer et al., 2004; Havea, Carr, & Creamer, 2004). The hydrophobic binding sites are involved in the hydrolysis by pepsin (Ahn, Cao, Yu, & Engen, 2013; Tang, 1963), thus enhanced accessibility of peptic cleavage sites would be expected in wet-heated BLG. Indeed, we observed a loss of hydrophobic regions upon gastric digestion, and this decrease of hydrophobicity was related to the degree of hydrolysis, as shown by wet-heated BLG in Fig. 3A and Fig. 4. The correlation between structural changes and gastric digestibility is indicated by Fig. 2 and Fig. S1, which show a clear relation between gastric digestibility and the structural change indicators-secondary structure, aggregation, and hydrophobicity. This is in line with our observation of increasing gastric digestibility correlating with the extent of structural modifications from native to low-temperature dry-heated, high-temperature dry-heated and wet-heated BLG as reported in our previous study (Deng et al., 2019). Higher peptic hydrolysis was observed for BLG samples heated under identical conditions in the presence of lactose, which is probably due to increased conformational changes induced by glycation (Chevalier, Chobert, Dalgalarrondo, Choiset, & Haertle, 2002; Morgan et al., 1999).

Not all previous studies on *in vitro* BLG intestinal digestion report similar results. For example, no significant digestion was observed in a study (Pinto et al., 2014), while in other studies, BLG could be partly digested by trypsin (up to 30%) or even completely digested by the end of intestinal digestion (Dupont et al., 2010). The variability in digestion protocols and activity of the used enzymes may have led to these different results. We observed complete digestion of all BLG samples after two hours of intestinal digestion (Fig. 1 and Table 1). The lower digestibility observed for intestinal digestion of the lactose-containing heated samples, especially in wet-heated BLG, may be explained by modification of cleavage sites as the amino acids involved in glycation, lysine and arginine, are also cleavage sites for trypsin. This is in agreement with a previous study reporting that an increased degree of glycation increases a protein's resistance to hydrolysis by lysine/arginine specific proteases (Deng, Wierenga, Schols, Sforza, & Gruppen, 2017). Undigested BLG was found in the jejunum one hour (and in some cases even two hours) after oral ingestion *in vivo* (Sanchon et al., 2018). In our study, the existence of undigested protein after 10 min of intestinal digestion was also observed. Due to this presence of undigested protein in the small intestine, the interaction between undigested BLG samples with intestinal cells is physiologically relevant.

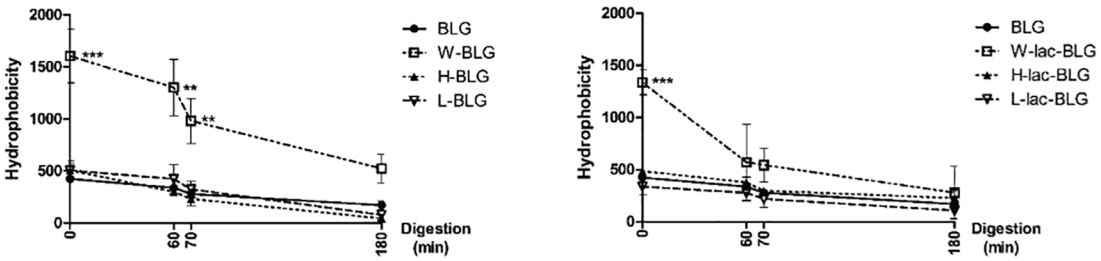

Fig. 4. Hydrophobicity of BLG wet-heated in the presence of lactose decreased significantly upon gastric digestion. BLG was treated and digested as described in the legend of Fig. 1 and levels of hydrophobicity were determined for each sample before digestion (0 min) or following gastric (60 min), 10 min of intestinal (70 min) and complete intestinal (180 min) digestion using ANS analysis. The lines represent averages ± standard deviation of n = 2 independent experiments. Statistically significant differences between heat-treated samples and native BLG for each step in the digestion were calculated with Dunnett's Test with: **p < 0.01; ***p < 0.001.

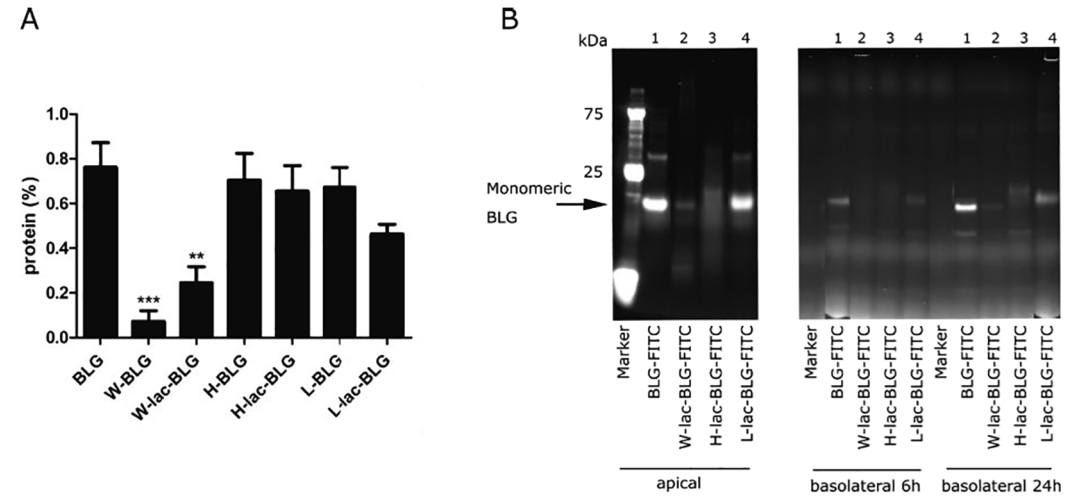

Fig. 5. Wet-heating of BLG significantly reduced translocation across a caco-2 monolayer. BLG was treated and digested as described in the legend of Fig. 1 and protein fractions were subsequently labelled with FITC. (A) Caco-2 cells grown for 21-days on transwell inserts were apically exposed to FITC-labelled samples for 6 h. The fluorescent signal in the basolateral compartment was analyzed using a fluorescence plate reader and indicated as percentage compared to the fluorescent signal applied to the apical compartment. Bars represent averages ± standard deviation of n = 3–4 independent experiments. Statistically significant differences between heat-treated samples and native BLG were calculated with Dunnett's Test with: *$p < 0.01$; ***$p < 0.001$. (B) Apical and concentrated basolateral medium after 6 and 24 h of incubation with medium or BLG samples were loaded onto SDS-PAGE gel and BLG-derived protein fractions were identified with a fluorescence scan using a Bio-Rad apparatus (a representative picture is depicted of n = 2 independent assays); Mw: molecular weight marker ranging from 5 to 250 kDa.

Translocation of BLG over the intestinal epithelial barrier can lead to the exposure of BLG to immune cells, which, in particular for heat-treated BLG, may lead to immune activation (Deng et al., 2019). Translocation over intestinal epithelial cells can occur either paracellular or transcellular and the latter was reported to be the main route for BLG translocation (Roth-Walter et al., 2008). Wet-heated BLG showed the lowest level of translocation through the Caco-2 layer (Fig. 5), which was also observed by another study (Perusko et al., 2018), although they did not study the effects of glycation in detail. The significant differences between the translocation of native and wet-heated BLG samples may be related to the formation of aggregates and structural modifications. A decrease in transport of particles through a Caco-2 monolayer was previously related to increasing particle size (Derakhshandeh, Hochhaus, & Dadashzadeh, 2011). Moreover, it was also observed that hydrophobic compounds had a lower transport rate across Caco-2 monolayer compared to hydrophilic compounds (Wirth, Bogner, & Gabor, 2001). Thus, the high hydrophobicity of wet-heated BLG might have further decreased its translocation through the Caco-2 monolayer.

5. Conclusion

As shown in our study, native BLG resists *in vitro* gastric and early intestinal digestion. Heat processing increased its digestibility to different extents, depending on the specific heating conditions. However, the undigested form of all processed samples still existed after 10 min of intestinal digestion. Furthermore, we demonstrated that undigested BLG was able to pass over an intestinal epithelial cell layer. This would allow the native or processed BLG to interact with local immune cells. Previously we demonstrated that wet-heating of BLG most strongly increased uptake by macrophages. However, here we demonstrate that wet-heated BLG was the most efficiently digested and also minimally translocated over the intestinal epithelial cells. We confirmed that heat-treatment affects BLG's digestibility and translocation over intestinal epithelial cells. Taken together with our previous findings (Deng et al., 2019), this indicates that BLG processing conditions and further *in vivo* analysis should be considered as there is a potential for low grade chronic immune activation by heat-treated BLG.

CRediT authorship contribution statement

Ying Deng: Methodology, Investigation, Validation, Visualization, Formal analysis, Writing - original draft. **Coen Govers:** Conceptualization, Writing - review & editing, Supervision. **Monic Tomassen:** Methodology. **Kasper Hettinga:** Conceptualization, Writing - review & editing, Supervision. **Harry J. Wichers:** Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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