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EVALUATION OF BOVINE MILK PROCESSING ON THE DIGESTIBILITY AND ALLERGENICITY OF MILK PROTEINS

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ABSTRACT

The objective of this study was to evaluate whether thermal processes applied in milk, such as pasteurization and UHT affect the protein digestibility leading to changes in the allergenic responses. Samples were subjected to a simulation of the human digestion and subsequently evaluated regarding protein cleavages and enzyme immunoassay for caseins and β-lactoglobulin immunogenicity. Among the different samples, protein digestibility was mainly affected in the gastric phase. α-lactalbumin and caseins showed high susceptibility to gastrointestinal enzymes, while a partial β-lactoglobulin resistance to pepsin was observed. Concerning *in vitro* allergenicity, a tendency of reduction was demonstrated in UHT and powdered milk samples after digestion in the stomach. Following the intestinal digestion, all milk samples presented low allergenicity, over 96% reduction of antibody binding. These data corroborates to the understanding of the effects of the world´s most used heat treatments in cow's milk protein digestibility and allergenicity.

1.Introduction

Bovine milk is an important source of proteins, lactose, calcium, vitamins and bioactive peptides (Villa *et al.*, 2018). In Western diets cow's milk consumption is also a habit among humans and its regular intake is associated with the prevention of several chronic diseases, including cardiovascular, diabetes, obesity and osteoporosis (Willett and Ludwig 2020). Despite its nutritional relevance and its whole on health preservation, bovine milk is on the list of the eight most allergenic foods (FAO and WHO, 2018). Currently, several allergenic epitopes have already been identified within the structure of the main milk proteins – caseins, alpha lactalbumin (α-La), beta-lactoglobulin (β-Lg). Cow's milk allergy affects 4% of children and 0.5% of adults worldwide, causing symptoms such as atopic dermatitis, acute urticaria, rhinitis, asthma exacerbation, vomiting, diarrhea and abdominal pain (Villa *et al.*, 2018, Willett and Ludwig, 2020). It is known that some food processes, including pasteurization and UHT process can modify the structure of proteins, either by glycosylation, Maillard reaction, aggregation or unfolding. These processes may alter the allergenic epitopes of proteins, influencing their binding to immunoglobulins, consequently modulating the immunological response (Bogahawaththa *et al.*, 2017, Bu *et al.*, 2013; Villa *et al.*, 2018). Different thermal processes such as pasteurization, ultra-high temperature (UHT) and UHT followed by atomization (milk powder) are used by milk industries to reduce microbiological contamination. However, the relation between these processes, especially UHT and atomization, with milk protein

allergenicity and digestibility is not fully understood, requiring further investigation (Bogahawaththa *et al*., 2017, Bu *et al*., 2013, Villa *et al*., 2018). Milk caseins are stable proteins when milk is treated by thermal processing, which generates a small attenuation of allergenicity and a slight increase in its digestibility. In contrast, the main whey proteins (α-La and β-Lg) are more susceptible to heat, especially when temperatures over 90 C° are applied as conformational changes leads to epitopes exposition and further destruction by the gastrointestinal enzymes (Rahaman *et al*., 2016, Villa *et al*., 2018). During protein digestion several chemical and enzymatic reactions occur, generating changes in the structure of proteins, which can either lower or increase their allergenicity. Not all proteins are fully cleaved in amino acids during digestion. Some of them are cleaved into larger peptides preserving allergenic epitopes, which may intensify certain immune system stimulations, such as IgE binding (Benede *et al*., 2014, Villa *et al*., 2018). Considering all mentioned above, the present study aimed to investigate different types of bovine milk processing – pasteurized, UHT and powdered (UHT followed by spray-drying atomization) – regarding the digestibility and allergenicity of milk proteins.

2. Materials and methods

2.1. Materials

Different commercially bovine milk, processed by pasteurization, UHT associated with homogenization and powder were selected. Samples of raw milk from local milk producers were also used for comparative purposes. The milk samples were selected from the dairy basin in the Southeast of Brazil, more specifically from Rio de Janeiro and Minas Gerais.

2.2. *In vitro* gastrointestinal digestion of milk samples

Digestion simulation was carried out in accordance to the international consensus on static in vitro digestion, Infogest (Brodkorb *et al*., 2019, Minekus *et al*., 2014). As recommended by the method for liquid foods, the salivary phase of the digestion was not performed. Therefore, simulated gastric fluid containing porcine pepsin (423 U.mg-1, Sigma-Aldrich, St. Louis, MO, USA) was added to the different milk samples. Gastric digestion occurred for 2 hours in a water bath (Banho Dubnoff NT 232, Novatecnica, Piracicaba, SP, Brazil) under constant agitation. To stop the reaction, the pH was adjusted to 7 with hydrochloric acid. Following to the intestinal phase, the solution was mixed with a simulated intestinal fluid containing porcine pancreatin (7.05 U.mg-1, Sigma-Aldrich, St. Louis, MO, USA) and bile (1.00 mmol.g-1, Sigma-Aldrich, St. Louis, MO, USA). The reaction occurred for 2 hours and it was stopped with ice bath. Samples were kept at -20°C until further analysis.

2.3. Degree of hydrolysis

The soluble protein content was quantified according to Bradford (1976), in a digital spectrophotometer SP-220 (Biospectro, Curitiba, PR, Brazil). The spectrophotometric measurement of aromatic amino acids was carried out according Goodwin and Morton (1946). A tyrosine standard curve was used and samples were read at 280nm. Results were analyzed by one-way ANOVA and submitted to the Tukey t-test in Microsoft Excel 2019 software with a significance level of $p = 0.05$.

2.4. Protein electrophoresis

One-dimensional protein electrophoresis was performed (Laemmli, 1970) using polyacrylamide gel. The stacking and running gels were prepared with 8% and 12% acrylamide solutions, respectively. Undigested and digested milk samples, as well as a wide molecular weight standard (Bio-Rad Laboratories, Inc, United States) were applied to the gels. The electrophoretic run was carried out in the Mini PROTEAN® Tetra Cell (Bio-Rad Laboratories, Inc, United States) at 100V for 2.5 hours. After running, the electrophoretic gels were fixed and stained in a solution containing acetic acid (10%), methanol (40%) and Coomassie Brillant Blue R 250 (1%) overnight.

2.5. Allergenicity of Milk Proteins

The allergenicity of milk samples and their digested products was determined by enzymatic immunoassay using sandwich ELISA kits for caseins and β-Lg (RIDACREEN FAST Milk, R-Biopharm AG, Darmstadt, Germany). The reaction was read in Multiskan FC (ThermoScentific, Waltham, MA, USA) at a wavelength of 450 nm. Casein and β-Lg concentrations were calculated by the RIDA®SOFT Win.net software (R-Biopharm AG, Darmstadt, Germany). The results correspond to the average of 4 experiments that were submitted to statistical analysis using Tukey's t-test in Microsoft Excel 2019 software with a significance level of p = 0.05 to compare the results.

3.Results and discussions

3.1. Milk Proteins Hydrolysis

Protein digestion began in the stomach by the activity of pepsin under acidic pH conditions. Pancreatic and intestinal proteases followed the digestion process, hydrolyzing the remaining protein fragments (Sah *et al*., 2016). In the present study, the soluble protein content dramatically reduced after the simulated gastric digestion achieving reductions of 24.3-, 19.5-, 25.7- and 27.3-times fold for raw, UHT, pasteurized and powdered samples, respectively (Figure 1A). After enteric digestion the soluble protein content of all bovine milk samples remained stable as the method is able to quantify proteins and peptides with molecular weight higher than 3 kDa. In contrast, no significant increase in the concentration of aromatic amino acids was observed from the undigested to gastric digested samples, showing that the peptic digestion was able to convert part of the protein into higher molecular weight peptides. Meanwhile, intestinal enzymes were able to release small peptides and amino acids and increments of 2.6-, 6.1-, 5.3- and 4.8-times fold for raw, UHT, pasteurized, and powdered milk samples, respectively, were observed after enteric digestion (Figure 1B).

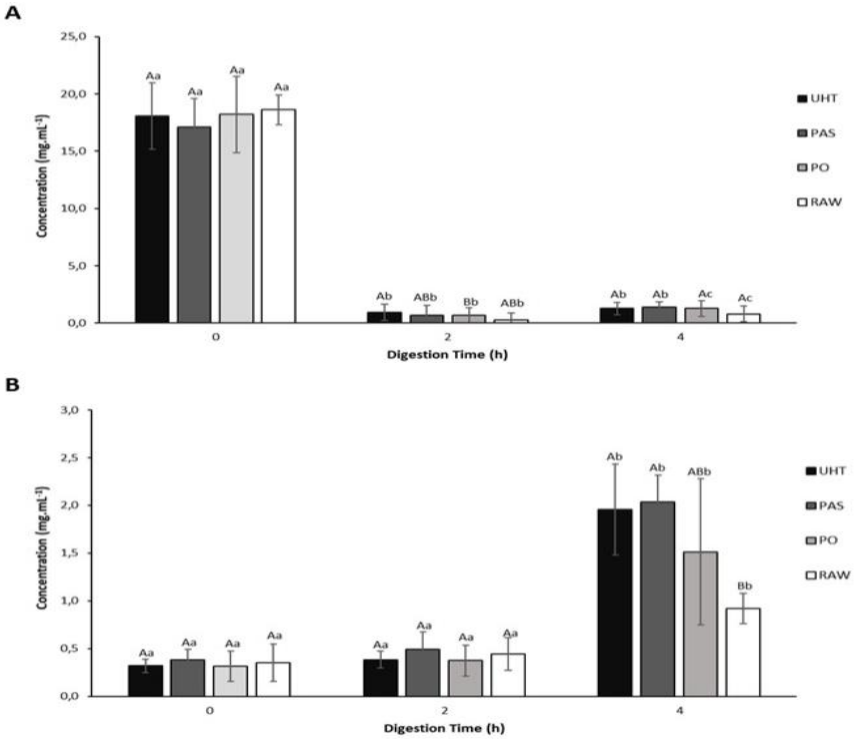

Figure 1. Soluble protein content (A) and aromatic amino acids (B) quantification of UHT, pasteurized (PAS), powdered (PO) and raw undigested and *in vitro* digested milk samples. Capital letters: significant difference between milk samples at the same time of digestion, $p < 0.05$; Lower-case letters: significant difference between digestion times in the same type of milk, $p < 0.05$.

3.2. Electrophoretic Profile

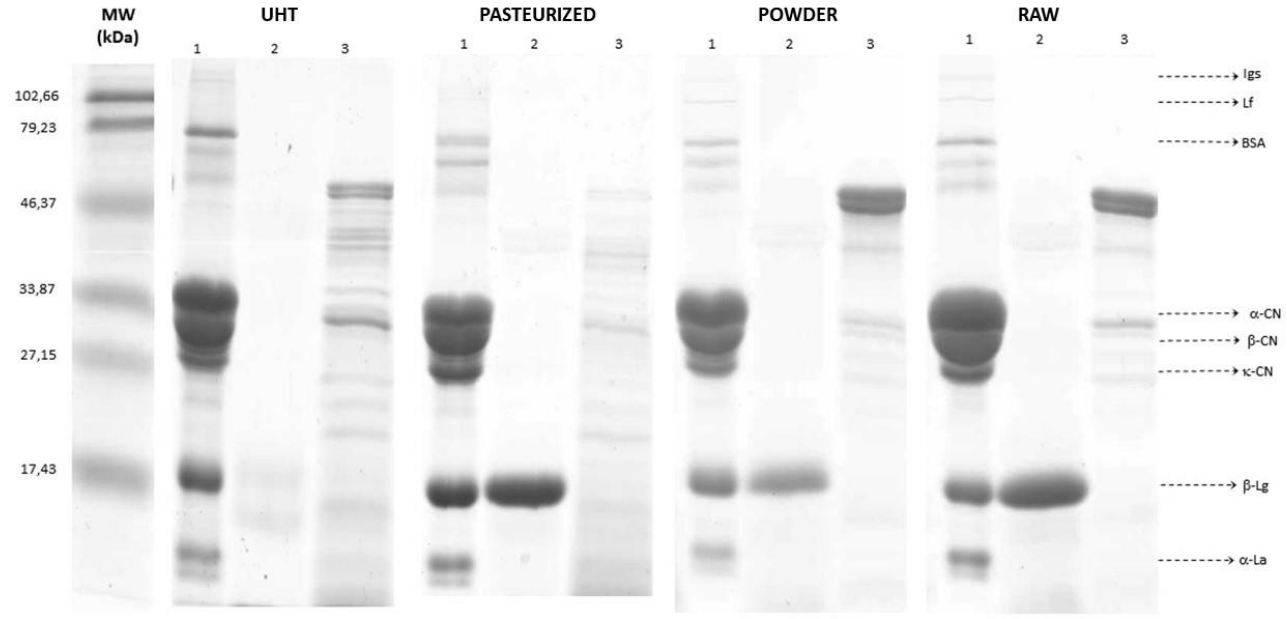

Figure 2. SDS-PAGE electrophoretic gel of UHT, pasteurized (PAS), powdered (PO) and raw milk during simulated digestion. MW: molecular weight; 1: undigested; 2: digested in the stomach; 3: digested in the small intestine; Igs: immunoglobulins; Lf: lactoferrin; BSA: bovine serum albumin.

The Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Figure 2) revealed that all cow's milk samples presented similar protein profiles, indicating that the different thermal processes applied did not affect their protein composition. According to Bouzerzour *et al.* (2012), approximately 77% of milk caseins are degraded before 30 minutes of digestion by pepsin and after the enteric phase of digestion they could no longer be found. Corroborating with this statement, in the present study the electrophoretic profile showed an intense reduction in casein bands for all milk samples after the gastric phase of digestion. Regarding whey proteins, the second most abundant protein in whey (α-La) was rapidly hydrolyzed by pepsin after the simulated gastric digestion for all milk treatments. Kopf-Bolanz *et al.* (2014) found similar results for raw, pasteurized and UHT whole milk and Mellinger-Silva *et al.* (2015) in pepsin hydrolysates of whey protein isolate. The susceptibility of α-La to pepsin can be attributed to conformational alterations occurred in α-La structure at low pH, exposing its hydrophobic interior and increasing its proteolysis by pepsin (Kamau *et al*., 2010, Nik *et al*., 2010). The major whey protein (β-Lg) presents a strong globular conformation at low pH, hiding pepsin's target amino acids (Ozorio *et al*., 2020). Bateman *et al.* (2010) and Ozorio *et al.* (2020) also reported a β-Lg resistance to simulated gastric digestion. In Figure 2 is possible to observe that in milk types treated with severe heat – UHT and powder – β-Lg was susceptible to pepsin, which is probably related to the thermal sensitivity of the β-Lg structure to high temperatures (Villa *et al*., 2018), exposing pepsin target sites. However, in milk samples that received mild or no heat treatment – pasteurized and raw milk, respectively – β-Lg was further hydrolyzed by intestinal enzymes. The susceptibility of β-Lg to enteric digestion can be associated to conformational alterations at pH above 7, uncovering amino acid residues

that are sensitive to intestinal enzymes (Ozorio *et al*., 2020).

3.3. Allergenicity of caseins and β-Lg

The sensitivity of an individual to a food allergen is very particular, explaining the lack of agreement regarding safe concentration for ingestion or minimum intake recommended to avoid allergic reactions (Villa *et al*., 2018). Figure 3 shows that prior in vitro digestion, all milk samples presented similar immunoreactivities to casein and β-Lg through ELISA tests. If IgE-mediated responses to milk proteins may cause symptoms usually right after ingestion or within the following 2h (Villa *et al*., 2018), all types of milk may generate immediate reactions in allergic people, since undigested samples presented high immunoreactivity. After gastric digestion, milk powder achieved the lowest allergenicity, followed by UHT, while pasteurized and raw milk showed similar profiles. Once β-Lg was completely and partially hydrolyzed in UHT and powdered milk (Figure 2), respectively, the most prominent reduction in the allergenicity of these both milk types may be related to a combination of severe heat treatment with pepsin digestion, as reported by Rahaman *et al*. (2016) and Villa *et al*. (2018). When compared to the intestinal digested, milk gastric digested samples presented higher allergenicity, which may be attributed to the preservation of allergenic epitopes in proteins and polypeptides not digested in this phase. Although in the present study the combination of alkali pH and intestinal enzymes had been able to drastically reduce the allergenicity in all cow's milk samples, some non-IgE-mediated allergic late reactions may still occur (Villa *et al*., 2018).

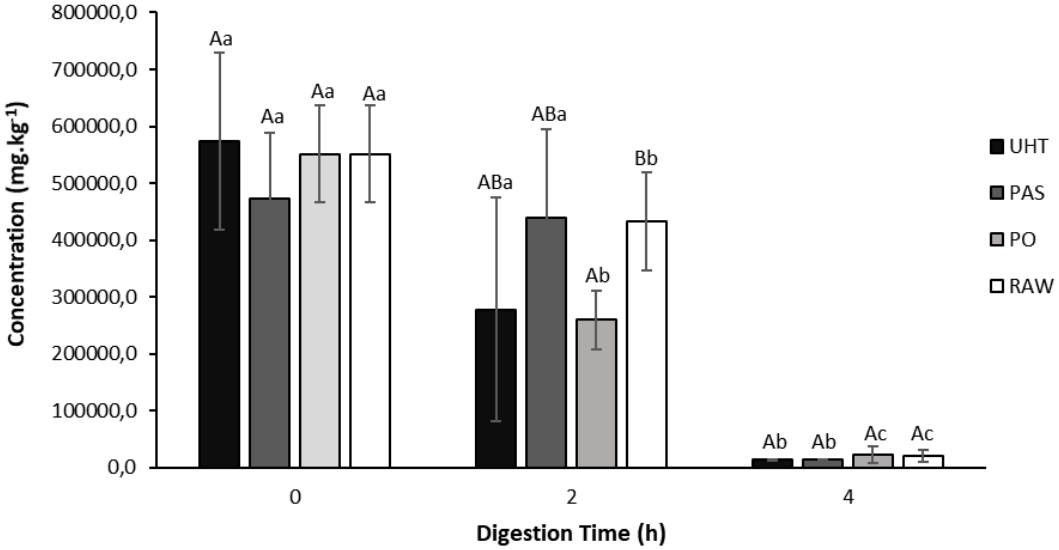

Figure 3. Concentration of casein and β-Lg in undigested and *in vitro* digested milk samples as an indicative of milk allergenicity. PAS – pasteurized milk; PO – powdered milk. Different capital letters: significant difference between milk samples at the same time of digestion; $p < 0.05$ Different lower-case letters: significant difference between digestion times in the same type of milk; $p < 0.05$.

4. Conclusions

Considering the results presented, undigested and in vitro digested milk samples evaluated showed similar degrees of hydrolysis. Through the electrophoretic gel it was possible to confirm the high susceptibility of cow's milk caseins to gastrointestinal digestion. The β-Lg of bovine milk types treated with intense heat (UHT and powder) showed more sensitivity to pepsin digestion than in raw and pasteurized ones. This can also be related to the tendential lower allergenicity of both UHT and powder milk gastric digests in comparison to pasteurized and raw gastric samples. Enteric enzymes could

hydrolyze the remaining proteins in all intestinal digested milk samples, which can be related to the small allergenicity observed for them. In this sense, the different thermal processes applied by industries to allow milk distribution worldwide besides providing a safe product, regarding microbiological contamination, also demonstrated small influence on the digestibility and allergenicity of the main milk proteins.

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