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Evaluation of different heat treatments applied to UHT milk added with transglutaminase

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Abstract

Milk is a nutritionally rich food, seen as a source of essential macronutrients and micronutrients. Lactose, which is milk's main carbohydrate, can be poorly metabolized by intolerant individuals, and it has led to the production of milk types with hydrolyzed lactose because they are more susceptible to non-enzymatic browning (Maillard reaction). Milk is subjected to heat treatments for preservation purposes, but it can cause undesirable changes in it. Strategies such as using stabilizing salts and transglutaminase have been explored as ways to minimize these effects. This enzyme catalyzes isopeptide bonds and improves milk technological quality. The aim of the present study was to investigate physicochemical and colorimetric parameters in whole milk, in whole milk added with transglutaminase, in lactose-free whole milk, and in lactose-free whole milk added with transglutaminase treated under different thermal conditions. Results have shown that refrigeration had a significant impact on hydrogen potential, ionic calcium, and particle size. In addition, transglutaminase addition increased thermal coagulation time in zero-lactose whole milk. Enzyme transglutaminase increased the browning index in most whole milk samples and reduced it in zero-lactose whole milk. Finally, this enzyme was able to reduce particle size.

Keywords: hydrolyzed milk; transglutaminase; heat stress.

Practical Application: This study breaks new ground by assessing the physicochemical and colorimetric parameters of UHT whole milk and lactose-free whole milk treated under different thermal conditions and added with transglutaminase. The tested thermal conditions were carried out at laboratory scale and were designed to test sample stress and to simulate extreme storage and transportation scenarios often faced in the dairy industry. It broadened the understanding of the role played by transglutaminase in dairy production, which opened room for its new industrial applications and for improvements in the processing and distribution chain.

1 INTRODUCTION

Milk supplies the body with all essential amino acids, besides being an excellent source of easily absorbable calcium because it lacks fibers, phytates, or oxalates, which hinder calcium assimilation (Guetouache et al., 2014). In addition to calcium, milk also comprises phosphorus, magnesium, zinc, selenium, and fat-soluble (A, D, and E) and water-soluble vitamins (B complex and vitamin C) that contribute to healthy diets (Cruz, 2016; Pereira, 2014). Proteins are among the most important macromolecules making up milk composition. These proteins account for essential functions in the human body, among them cell repairing, building and repairing muscles and bones, and regulating several biochemical mechanisms, despite their technological and functional properties (Cruz, 2016; Krishna et al., 2021; Stobiecka et al., 2022).

Lactose is the main carbohydrate in milk. It is made of two monosaccharides, namely glucose and galactose, gathered by a β-1,4 glycosidic bond (Romero-Velarde et al., 2019). β-*galactosidase* (lactase) boosts lactose hydrolysis; it is found in the humans' small intestine, and its absence or reduced activity allows lactose to pass intact to the colon, where it is fermented. This process results in gases accountable for flatulence, abdominal distension, and for the production of acids that, together with increased osmotic pressure, cause diarrhea. This clinical condition is known as lactose intolerance (Francisquini et al., 2024; Singh et al., 2024). Therefore, in order to minimize these gastrointestinal symptoms, it is recommended to include food with low lactose content. However, these products are prone to non-enzymatic browning (Maillard reaction), and it can lead to consequences such as carcinogenic by-product production,

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which affects nutritional value and sensory properties (Bi et al., 2023; Singh et al., 2024).

Milk is subjected to heat treatment for public health and conservation reasons. The aim of this treatment is to rule out possible pathogens, as well as to deteriorate microorganisms, enzymes, and to extend shelf life. The most commonly used methods include pasteurization (72–75°C for 15–20 s), sterilization (115C–120°C for 20–30 min), or ultra-high temperature treatment—UHT (135–140°C for a few seconds). Different changes can be observed depending on the heat treatment type, among them micellar aggregation, Maillard reaction, casein dephosphorylation, protein denaturation, among others (Anema, 2017; Da Silva Araújo et al., 2021; Elhasan et al., 2017; Gaucheron, 2005; Holt, 1995; Silva, 2003). Different strategies are used to minimize heat treatment effects, like stabilizing salts addition. More recently, it is possible to observe the use of the enzyme transglutaminase (Singh, J. et al., 2021).

Enzyme transglutaminase can catalyze the acyl transfer reaction. It consists of acyl transfer between the γ-carboxyamide group of a glutamine residue (acyl group donor) in a protein's peptide chain and a primary amine in the presence of the amino acid lysine. This process results in intermolecular and intramolecular bonds (Gharibzahedi et al., 2018; Velazquez-Dominguez et al., 2023). Water becomes the receptor in the absence of primary amines, and it opens room for deamidation to happen, which converts glutamine residues into glutamic acid (Vasić et al., 2023). This enzymatic action requires specific temperature conditions (ideal range from 40 to 60°C), pH (from 5 to 8), and substrate. This action ends due to denaturation or when the active sites are no longer available. The amount of enzyme used in each food depends on the protein type and amount (Francisquini et al., 2024; Mostafa, 2020; Salunke & Metzger, 2023).

With respect to regulatory aspects, enzyme transglutaminase is acknowledged as safe by the Food and Drug Administration and the Food Sanitation Act, which are used as references in the United States and Japan, respectively (Francisquini et al., 2024). Transglutaminase use in Brazil is regulated by Collegiate Board Resolution n. 585, from December 10, 2021. This resolution provides for enzymes and enzyme preparations to be used as adjuncts to technology applied to produce food intended for human consumption. There is no need to include this enzyme in the list of ingredients because this is seen as an addition to the manufacturing technology (Brasil, 2022; Francisquini et al., 2024).

Accordingly, it is possible to use enzyme transglutaminase in different food matrices and achieve different functional features in different food proteins, namely water retention, gel formation, foam formation, viscosity, elasticity, emulsification capacity, gelation, and thermal stability (Bönisch et al., 2006; Li et al, 2021; Miwa, 2020). Therefore, the aim of the present study was to investigate the physicochemical and colorimetric parameters of heat-treated UHT whole milk and zero-lactose whole milk added with transglutaminase.

1.1 Relevance of the work

Enzyme transglutaminase has been assessed for its ability to modify milk proteins by influencing their stability and functional properties. However, the interaction between this enzyme and different heat treatments applied to ultra-high temperature (UHT) milk remains little explored. The aim of the present study is to investigate transglutaminase effects under different thermal conditions by analyzing physicochemical and structural parameters. The results will help in better understanding the impact of this enzyme on UHT milk stability, which will help the dairy industry develop products with higher quality and longer shelf life.

2 MATERIALS AND METHODS

2.1 Sample preparation

UHT whole milk and lactose-free whole milk samples from the same brand were purchased. The milk types were divided into four groups: whole milk (WM), whole milk added with transglutaminase (WMTG), lactose-free whole milk (WMH), and lactose-free whole milk added with transglutaminase (WMHTG). The aliquot of 1,000 g sample of each group was weighed. The WMTG and WMHTG groups were added with 3.86 and 3.60 U g^{-1} transglutaminase protein, respectively, and left to stir in a digital mixer (IKa RW 20) for 5 ± 2 min. The enzyme was supplied by Ajinomoto (ACTIVA WM, 80 to 120 units per gram). The pH of all the groups was adjusted to 6.6 ± 0.05 with the aid of a portable digital pH meter coupled to a Gehaka PG 1400 microprocessor. Slowly, 1 M sodium hydroxide (NaOH) by Sigma-Aldrich (Missouri, USA) was added to the samples. Finally, all samples were subjected to refrigeration heat treatment at 8°C ± 2°C for 24 h.

2.2 Experimental planning

WM, WMTG, WMH, and WMHTG samples were subdivided into five groups (F_{0C}; $F_{0.6}$; $F_{0.7}$; F_{44}; and F_{77}) after the storage time was over. The tests were applied to three repetitions. The control group was removed from refrigeration and taken for further analysis (F_{0C}) right away. The other four groups were subjected to different heat treatment conditions, namely oven for 5 h at 95°C ± 2°C ($F_{0.6}$), oven at 95°C ± 2°C for 7 h ($F_{0.7}$), autoclave at 121°C ± 2°C for 25 min (F_{44}), and autoclave at 121°C ± 2°C for 60 min (F_{77}). JF MEDICAL autoclave model AV50 was used in the experiment, as well as the M.S.MISTURA oven model MSM 512/100.

The five groups were named based on the F_0 value, which corresponds to the equivalent exposure time at 121.11°C based on an ideal microorganism recording thermal destruction coefficient equals 10°C (Pistolesi & Mascherpa, 2015). The treated groups recorded the following F_0 values: 0C, 0.6, 0.7, 44, and 77. Autoclave and oven temperatures during heat treatment were monitored with the EL-USB-T-PRO sensor (ranging from -40 to 125°C). Data collected in the Easylog USB software generated the tables used to calculate F_0. This calculation was carried out in the lethality calculator by using Simpson's rule. This method was used to standardize the heat treatments (Mullan, 2018).

2.3 Analysis

All analyses were carried out in duplicate at room temperature (22°C ± 2°C).

2.3.1 Hydrogen Potential

A PG1400 pH meter (Gehaka, São Paulo, Brazil) was used in the analysis. This equipment was calibrated with standard pH solutions (4.0 and 7.0).

2.3.2 Ionic calcium

As described in Singh et al. (2019), calcium levels were measured with a LAQUAtwin portable calcium selective ion electrode (Horiba Instruments, Kyoto, Japan). The electrode was calibrated with 3.74 mmol L^{-1} (150 ppm) and 49.90 mmol L^{-1} (2,000 ppm) calcium standard solutions before each experiment.

2.3.3 Particle size distribution

The LS 13,320 laser diffraction particle size analyzer (Beckman Coulter, California, United States) with an aqueous liquid module was used in this analysis. The samples were slowly added to the equipment's reservoir until reaching the minimum obscuration level. Results were expressed as Dv90 and Dv10. In total, 90% of particles were below the hydrodynamic size Dv90, and 10% of them were below Dv10.

2.3.4 Colorimetry and Browning index

A CR-400 chroma meter (KONICA MINOLTA) was used in the color analysis. It provided the direct reflectance reading of coordinates L* (luminosity), a* (positive = red, negative = green) and b* (positive = yellow, negative = blue). These parameters were used in Equations 1 and 2 to calculate the BI (Maskan, 2001).

$$BI=([100\ (x-0.31)])/0.17 \quad (1)$$

$$\text{Wherein } x=((a^*+1.75L^*))/((5.645L^*+a^*-3.012b^*)) \quad (2)$$

2.3.5 Heat coagulation time

A glass vial filled with a 10 mL of sample was placed on a shaker and immersed in an oil bath at 155°C ± 2°C for the analysis. The flask was positioned in the oil bath in such a way that the sample level was equal to the oil bath level. It was done to make coagulation-moment visualization easier. HCT is the time elapsed between placing the samples in the oil bath and the emergence of the first visible sign of coagulation (Francisquini et al., 2023).

2.3.6 Alcohol number

The aliquot of 10 mL of milk was transferred to a 100 mL Erlenmeyer flask for the analysis. Ethanol (96%) was titrated in an automatic burette to the point where precipitate formation was observed. The alcohol number corresponds to the amount of 96% ethanol needed to cause precipitation in 10 mL of milk (Tarapatskyy et al., 2019).

2.4 Statistical treatment

Means recorded for the collected data were compared through Tukey's test at a 0.05 significance level in R® software (The R Project for Statistical Computing), version 4.4.1.

3 RESULTS AND DISCUSSION

Mean results recorded for the assessed WM and WMH sample parameters in the refrigeration process, with and without transglutaminase addition, are shown in Figure 1. Results of samples from the same starting milk were compared.

WM and WMH samples' pH values significantly increased after 24-h refrigeration ($p < .05$) regardless of transglutaminase presence. This increase can be related to calcium phosphate content reduction at low temperatures (Karlsson et al., 2019; Li et al., 2020; Ranvir et al., 2020; Walstra et al., 2005; Wang & Ma, 2020). A significant ionic calcium decrease was also observed after refrigeration, except for WMTG. It likely happened because of a shift in balance between the free and bound forms of calcium (Geerts et al., 1983; Tsioulpas et al., 2007; Tsioulpas et al., 2010).

According to Figure 1c, Dv90 values showed significant reduction ($p < .05$) in particle size after refrigeration in all samples, except for WMTG. It is important to observe that ionic calcium and Dv90 decrease was not statistically significant ($p > .05$) when WMTG samples were compared before and after 24-h refrigeration. This finding points out stability in these parameters during refrigeration. These findings corroborate previous studies that have highlighted the enzyme transglutaminase's ability to catalyze reactions between proteins, mainly casein, which results in a more cohesive micellar structure fundamental for micelle stability (Chen & Hsieh, 2016; Duerasch et al., 2018; Ye & Harte, 2013). Gaspar and Góes-Favoni (2015) showed that the enzyme transglutaminase can prevent micelles from disintegrating and maintain ionic calcium concentration.

According to the figure, Dv10 values highlighted no significant difference ($p > .05$) among WM, WMH, and WMHTG before and after refrigeration. However, after refrigeration, there was a significant Dv10 reduction in WMTG (0.15 μm) in comparison to WM (0.19 μm). Previous studies, such as that by Velazquez-Dominguez et al. (2023), pointed out particle size variations in milk due to discrepancies in experimental conditions between studies. However, it is widely accepted that the mean diameter of casein micelles, which comprise approximately 80% of milk proteins, is close to 0.2 μm. Casein is an efficient substrate for transglutaminase action due to its flexible structure and availability of amino acids lysine and glutamine, which make the formation of intermicellar and intramicellar bonds easier. This crosslinking mechanism helps to reduce particle size (Puri et al., 2021; Silva et al., 2019; Velazquez-Dominguez et al., 2023).

In Figure 2, the mean HCT and alcohol number results recorded for WM and WMH samples with and without transglutaminase addition are shown.

HCT results have shown a statistically significant increase ($p < .05$) from 11.75 min to 18.84 min in it, respectively, when WMH was compared to WMHTG (Figure 2a). Tarapatskyy et al. (2019) showed that the enzyme transglutaminase in raw and

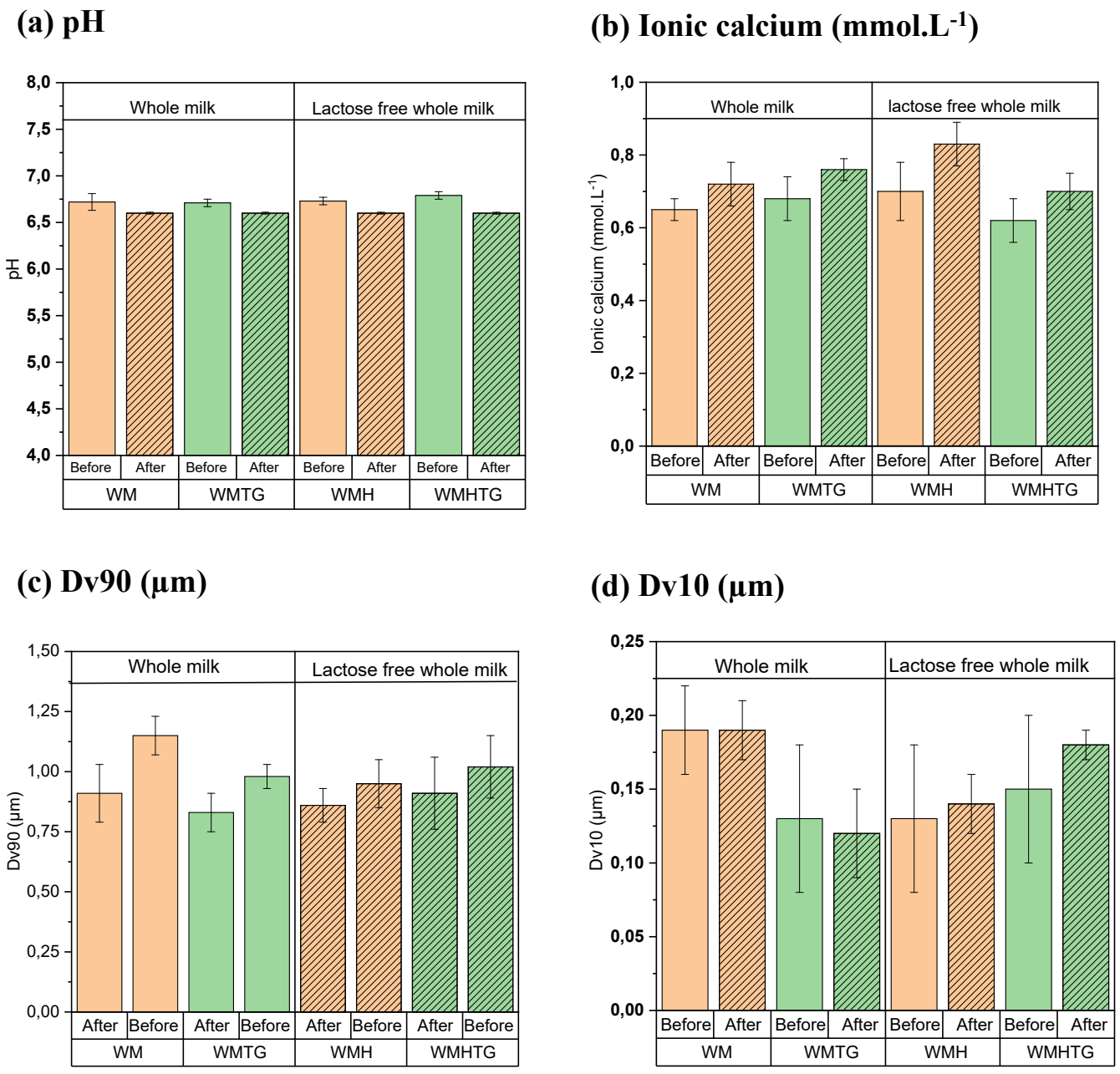

Figure 1. **(a)** WM, WMTG, WMH, and WMHTG samples' pH, **(b)** ionic calcium (mmol L^{-1}), **(c)** Dv90, and **(d)** Dv10 before refrigeration at 22°C ± 2°C and 24 h after refrigeration at 8°C ± 2°C.

pasteurized whole milk did not have a significant impact on thermal stability measured as thermal coagulation time. However, there are no reports in the scientific literature about HCT analysis applied to UHT whole milk, with and without hydrolyzed lactose, added with transglutaminase. Therefore, it can be seen that heat increases to 155°C ± 2°C in the analysis, affecting lactose-free whole milk thermal stability. Furthermore, the enzyme transglutaminase could increase thermal coagulation time and make the product more stable (O'Sullivan et al., 2002; Ritota et al., 2017).

Alcohol number (Figure 2b) results have shown a statistically significant difference ($p < .05$) between WM (mean value = 10.9 mL) and WMTG (mean value = 18.86 mL) samples. Although there are no reports in the literature about running this test with UHT whole milk added or not with transglutaminase, evidence suggests that this enzyme contributes to micellar integrity through intramicellar crosslinking in the core of casein micelles, since it reinforces their structure through covalent bonds (Fagnani et al., 2018; Raak & Corredig, 2022; Velazquez-Dominguez et al., 2023).

Figure 3 illustrates the results recorded for the assessed WM and WMH parameters, with and without transglutaminase addition, under different heat treatment conditions.

No statistically significant differences were observed in any heat treatment based on the results shown in Figures 3a and 3b.

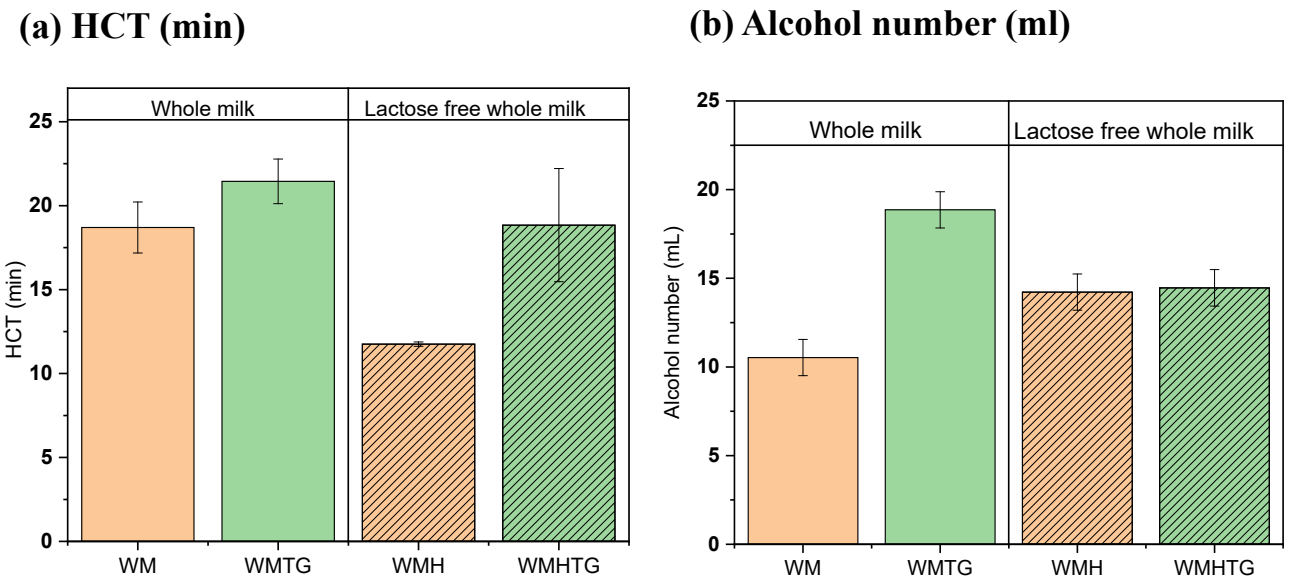

Figure 2. **(a)** WM, WMTG, WMH, and WMHTG samples' HCT (min) and **(b)** alcohol number (mL), after 24-h refrigeration at (8°C ± 2°C).

Enzyme transglutaminase was only able to reduce ionic calcium values from 1.09 to 0.84 mmol L^{-1}, respectively, when the WMH sample was compared to WMHTG (Figures 3a and 3b).

BI results shown in Figure 3e pointed to an upward trend when WM samples were compared to WMTG samples. According to this finding, enzyme transglutaminase overall helped achieve increased browning in these samples. According to results in Figure 3f, transglutaminase addition was effective in reducing WMH's IE value for WMH when it was compared to WMH.

IE variability can be explained by the different thermal conditions applied to the assessed samples. According to Fox (1989), the time/temperature binomial influences structural and physical-chemical changes in the assessed matrix when milk is subjected to different heat treatments. In addition, the milk and lactose-free whole milk matrices are different because the presence of monosaccharides glucose and galactose in hydrolyzed milk influences non-enzymatic browning differently, since the presence of these reducing sugars is associated with intensified Maillard reaction (Francisquini et al., 2024; Shibao & Bastos, 2011).

Studies that use enzyme transglutaminase and assess colorimetry parameters available in literature focus on finding its effects on hydrolyzed skimmed milk powder or on ice cream treated with transglutaminase plus lactase (Francisquini et al., 2024; Pereira et al., 2020). Thus, there are no studies available about the effects of different heat treatments under the herein tested conditions.

Figure 4 shows results recorded for WM and WMH sample parameters with and without transglutaminase addition under different heat treatment conditions.

Results shown in Figures 4a and 4b point out that, overall, enzyme transglutaminase helps to reduce particle size. Mean Dv90 results recorded for the WM sample are shown in Figure 4c. A significant difference was observed ($p < .05$) in treatment F_{44}, which showed a mean WM value equal to 0.83 µm and a mean WMTG value equal to 1.07 µm. Treatment F_{77} accounted for a significant reduction ($p < .05$) when WM (mean value = 1.14 µm) was compared to WMTG (mean value = 0.92 µm). Figure 4d shows the Dv90 results recorded for the WMH sample. WMH recorded a mean value of 1.31 µm and WMHTG mean value of 0.89 µm under treatment F_{44}, as well as statistically significant reduction in it ($p < .05$). According to the analysis applied for particle size values under the F_{77} treatment, the WMH sample recorded a significantly higher mean value (22.06 µm) than WMHTG (0.93 µm). This finding shows that thermal stress under the F_{77} treatment conditions destabilized the protein matrix, which resulted in larger aggregates in the WMH sample. However, transglutaminase addition had a remarkable impact on matrix stabilization, and it was proven by the significant reduction in particle size in the WMHTG sample.

Figure 5 shows the principal component analyses separated into WM and WMH.

Three samples (F_{0c}-WM, F_{0c}-WMTG, and $F_{0.7}$-WMTG) were grouped together (red color) in Figure 5a. Their pH (ranging from 6.56 to 6.60) was the parameter responsible. On the other hand, samples $F_{0.6}$-WM and $F_{0.6}$-WMTG formed a different group (black color), which was also determined by pH (ranging from 6.62 to 6.64).

The first grouping (blue color) included F_{0C}-WMH and F_{0C}-WMHTG samples with pH ranging from 6.60 to 6.61 (Figure 5b). The second group (yellow color) included samples F_{44}-LH, F_{44}-LHTG, $F_{0.7}$-LH, $F_{0.7}$-LHTG, and F_{77}-LHTG and was determined by ionic calcium (0.84 to 1.19 mmol L^{-1}) and browning index (50.71 to 66.14). The third grouping (green) highlighted the F_{77}-WMH sample, which showed a marked increase in particle size (Dv10 and Dv90). Significant particle

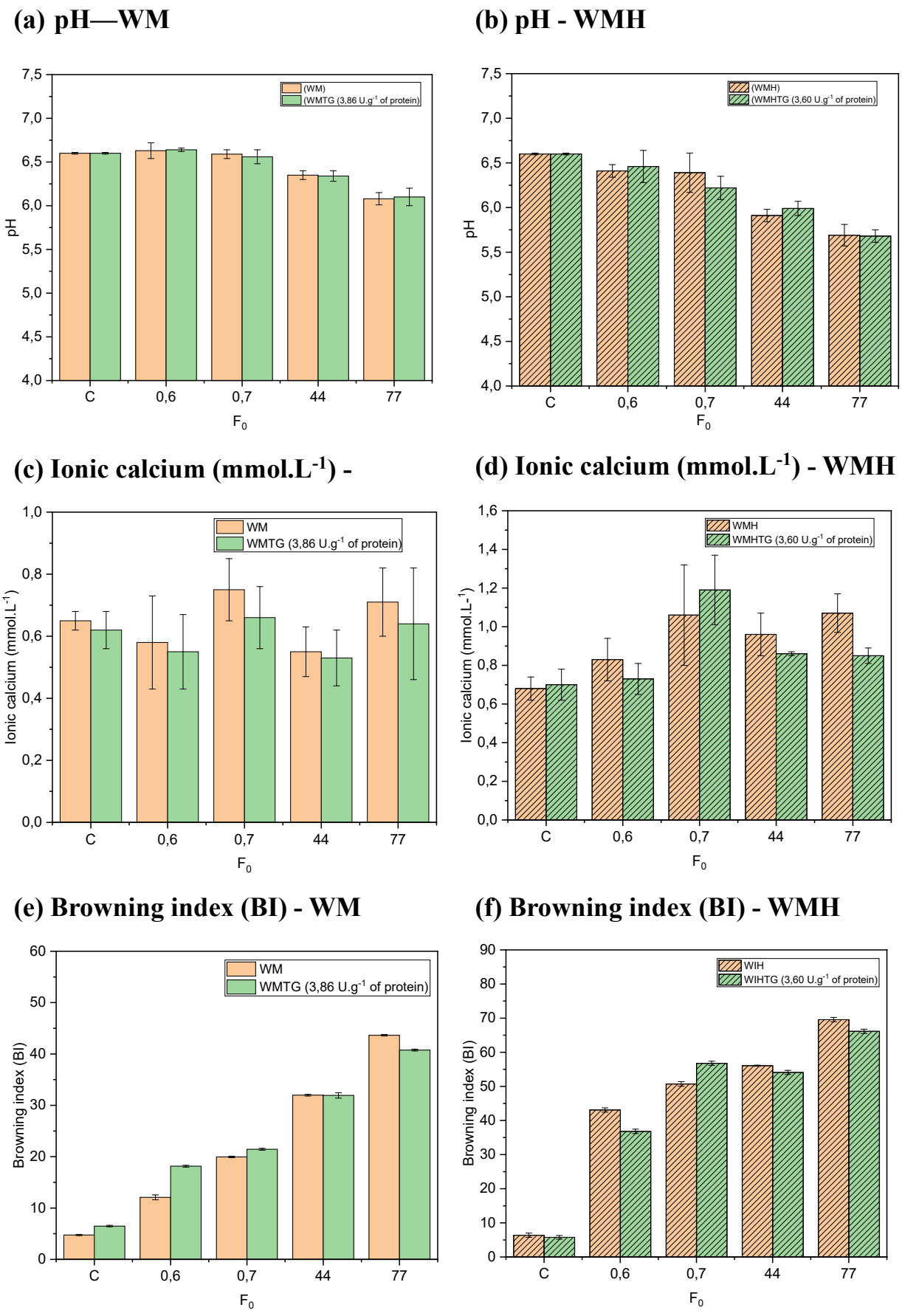

Figure 3. WM, WMTG, WMH, and WMHTG samples' **(a, b)** pH, **(c, d)** ionic calcium (mmol L^{-1}), **(e, f)** browning index (BI).

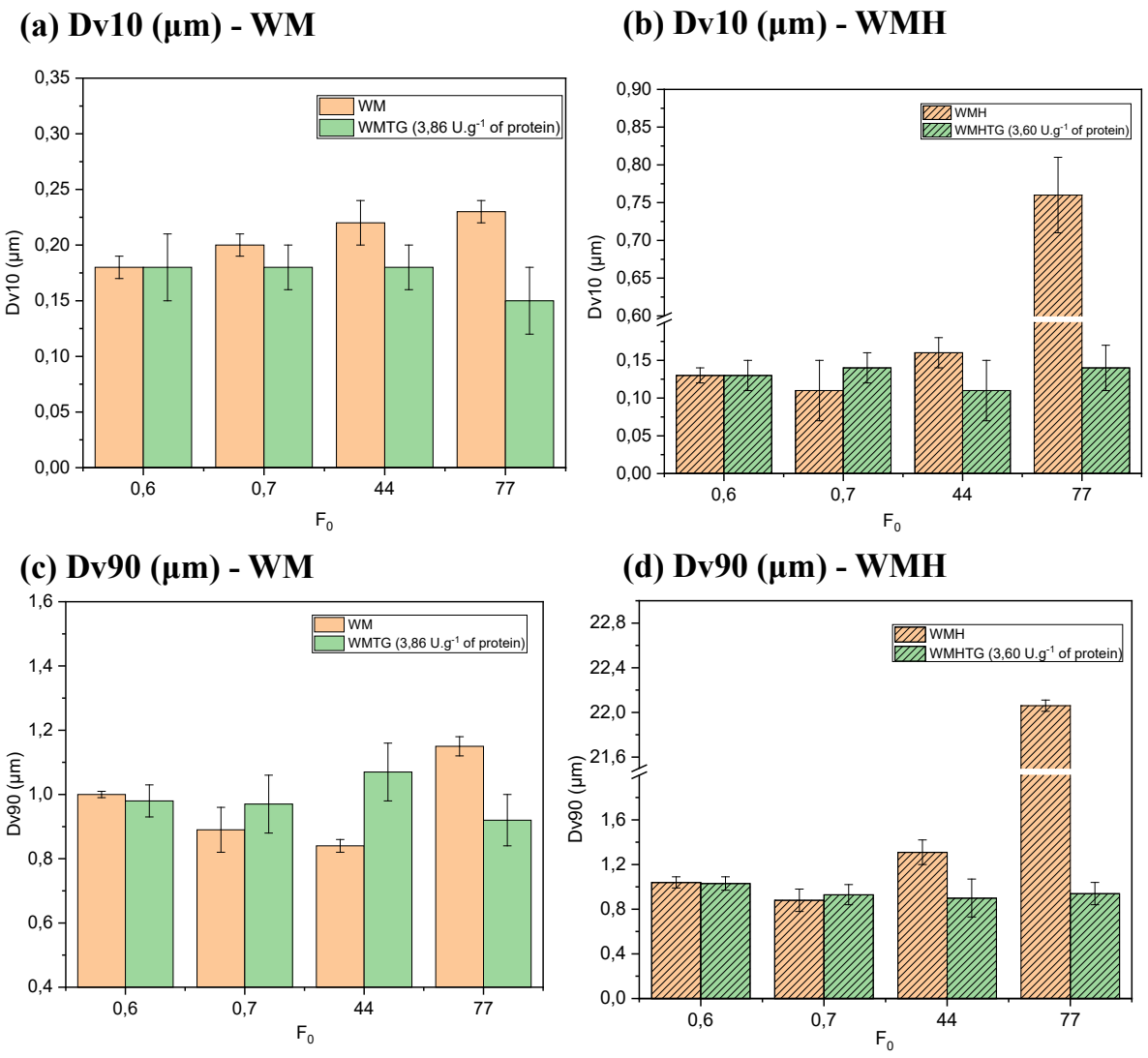

Figure 4. **(a, b)** Dv10 and **(c, d)** Dv90 recorded for WM, WMTG, WMH, and WMHTG samples heat-treated with the following F_0: $F_{0,6}$; $F_{0,7}$; F_{44}; and F_{77}.

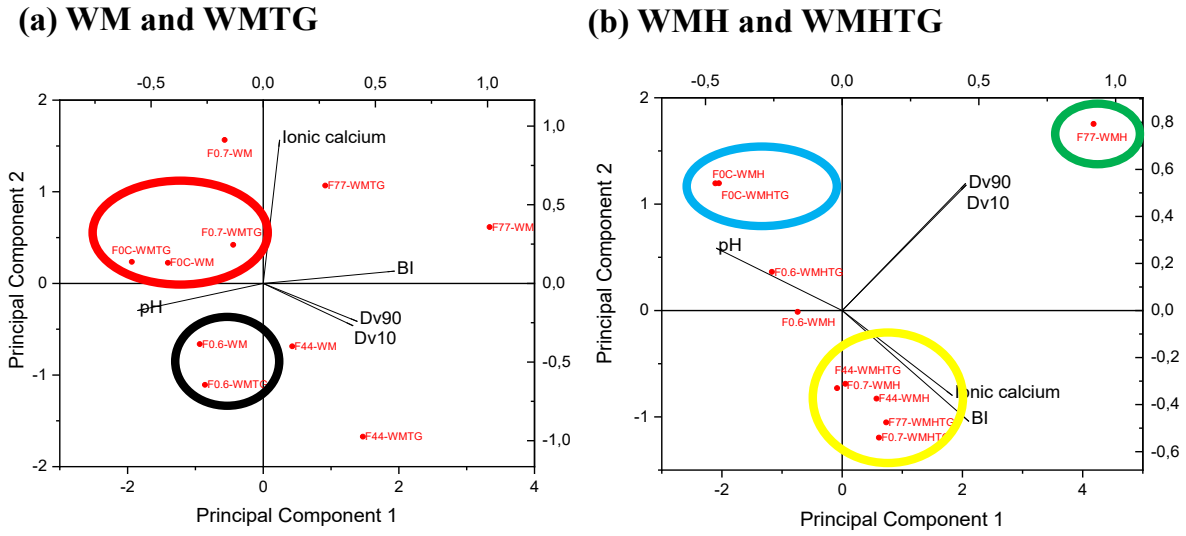

Figure 5. Principal component analysis of parameters pH, ionic calcium, IE, Dv10, and Dv90 recorded for **(a)** WM and WMTG and **(b)** WMH and WMHTG samples heat-treated with the following F_0: $F_{0c;}$ $F_{0.6}$; $F_{0.7}$; F_{44}; and F_{77}.

size reduction could be seen when the same sample was assessed with transglutaminase addition, and this finding corroborates results in Figure 4. These findings point out that the enzyme transglutaminase plays a key role in reducing the particle size of UHT whole milk with and without hydrolyzed lactose.

4 CONCLUSION

Results allowed observing that refrigeration has a significant effect on physicochemical parameters such as pH, ionic calcium, Dv10, and Dv90. Enzyme transglutaminase addition helped to stabilize parameters, ionic calcium, and Dv90 in UHT whole milk samples. Furthermore, particle size reduction expressed by Dv10 showed this enzyme's ability to catalyze peptide bonds, mainly at the size range corresponding to caseins.

UHT whole milk samples showed no significant changes in thermal stability (HCT). However, transglutaminase addition increased thermal coagulation time in UHT zero-lactose whole milk since it provided greater stability to it. In addition, enzyme transglutaminase contributed to micellar integrity, as evidenced by the alcohol number test applied to UHT whole milk. Enzyme transglutaminase increased the browning index in most UHT whole milk samples. On the other hand, this enzyme was effective in reducing this parameter in UHT zero-lactose whole milk.

Overall, particle size distribution was significantly influenced by the presence of the enzyme transglutaminase, which stood out for its ability to reduce particle size. This effect was mostly clear in UHT zero-lactose whole milk treated under condition F_{77}, since the enzyme accounted for a significant reduction, which highlighted its efficiency in causing structural modification in particles. Therefore, results reported in this study highlight the need for further studies about enzyme transglutaminase's influence on dairy products.

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