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Enrichment of tiger nut milk with microbial transglutaminase cross-linked protein improves the physico-chemical properties of the fermented system.

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Abstract

Milk proteins cross-linked with microbial transglutaminase were investigated for their potential to improve the microbiological and physico-chemical properties of fermented tiger nut milk. Fermented systems with cross-linked proteins did not affect S. *thermophilus* viable counts but decreased that of *L. delbrueckii* ssp. *bulgaricus* compared to the untreated protein systems. Systems with cross-linked proteins showed shorter microbial lag time and a higher pH reduction rates during fermentation. During storage of the fermented product, viable counts of *L. delbrueckii* ssp. *bulgaricus* decreased faster than that of *S. thermophilus*, and systems with cross-linked proteins revealed a lower decrease in *L. delbrueckii* ssp. *bulgaricus* cell counts compared to untreated proteins during 15 d. Products from cross-linked sodium caseinate or whey protein showed 16.4 fold and 3.6 fold increase in viscosity, and approx. 30 % and 36 % decrease in syneresis compared to their untreated counterparts, respectively. The addition of proteins to tiger nut milk improved the lightness of the fermented product and minimized lightness decrease during storage, and casein cross-linking further improved lightness. The enrichment of tiger nut milk with cross-linked protein has therefore a large potential for improving the physical characteristics of fermented tiger nut milk.

Keywords: Tiger nut milk, enzymatically cross-linked protein, fermentation, viscosity, syneresis.

1. Introduction

Lactic acid fermentation of tiger nut (*Cyperus esculentus* L.) aqueous extracts, also denoted as tiger nut milk (TNM), is known to give lactose-free, sweet-sour products that might serve as important source of food nutrients (Akoma, Elekwa, Afodunrinbi, & Onyeukwu, 2000; Wakil, Ayenuro, & Oyinola, 2014). However, lactic acid fermentation of plain tiger nut milk leads to products with low viscosity and high susceptibility to phase separation (Kizzie-Hayford, Jaros, Zahn, & Rohm, 2016), which adversely affects consumer acceptance of the product (Akoma *et al*. 2000). Our recent report revealed that the enrichment of TNM with milk proteins and subsequent lactic acid fermentation resulted in yogurt-like products with acceptable textural and sensory properties (Kizzie-Hayford *et al.*, 2016). Tiger nut milk itself shows a protein content as low as < 1 g/100 g, which does not allow the fermented product to build up a sufficient texture (Kizzie-Hayford *et al.*, 2016). Thus, addition of milk proteins is necessary for enhancing texture and sensory properties of the fermented product, and may help to improve the protein supply of consumers.

For marketing purposes, additional knowledge on the storage properties of fermented TNM is essential to monitor and predict product quality. Depending on product composition, changes in the physico-chemical attributes of the fermented system might occur during storage because of microbial imbalances, post acidification and syneresis (MacBean, 2009). Exemplarily, syneresis might have profound effects on the storage quality of fermented systems as even plain TNM exhibits a limited colloidal stability (Kizzie-Hayford, Jaros, Schneider, & Rohm, 2015). This contributes to appearance and texture defects of the fermented system, and impacts consumer acceptance (Walstra, Geurts, & Wouters, 2006). Microbial imbalances and post

41 acidification contribute to textural defects, promote wheying-off and might cause excessive
42 sourness of yoghurt (Yildiz, 2010).

43 For stirred yogurt, an increase in viscosity and a reduction of syneresis during storage
44 were observed after pre-treatment of the base cow milk with microbial transglutaminase
45 (mTGase, EC 2.3.2.13; Jaros, Heidig, & Rohm, 2007). This enzyme is mainly produced by
46 *Streptomyces mobaraensis*, and commercially available for the food industry. It cross-links
47 proteins through the formation of isopeptide bonds between protein-bound lysine and glutamine
48 residues, which improves the texture of acid protein gels made thereof (Jaros, Partschefeld,
49 Henle, & Rohm, 2006; Rohm, Ullrich, Schmidt, Löbner, & Jaros, 2014). Pre-treatment of cow
50 milk with mTGase was also reported to prolong fermentation time, increase gel strength and
51 reduce post acidification in set-style yogurt (Lorenzen, Neve, Mautner, & Schlimme, 2002; Ozer
52 *et al.*, 2007). In contrast, Romeih, Abdel-Hamid, & Awad (2014) showed that mTGase had no
53 effect on the acidification rate of buffalo skim-milk. Instead, the simultaneous addition of
54 mTGase and butter milk powder to buffalo skim-milk resulted in shorter fermentation time.
55 Effects of mTGase treatment on microbial acidification might therefore depend on the type of
56 fermentation substrate.

57 Currently, there is no evidence in the literature regarding the effect of mTGase cross-
58 linked proteins on the microbiological properties of fermented tiger nut milk. Exploring the
59 potential of mTGase cross-linked proteins for improving the physico-chemical properties of
60 fermented TNM is novel, as it might lead to products with enhanced texture and improved
61 storage properties (Kizzie-Hayford *et al.*, 2016). Therefore, the present study investigates the
62 effects of mTGase-modified proteins added to tiger nut milk on the microbiological and physico-
63 chemical properties during fermentation and storage.

2. Materials and methods

2.1. Materials

Tiger nuts were supplied by farmers at Twifo Praso in the Central Region of Ghana, and were prepared by cleaning and drying, and subsequently stored as described previously (Kizzie-Hayford *et al*., 2015). Sodium caseinate was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), xanthan gum from Cargill France SAS (Saint-Germain-en-Laye, France) and whey protein isolate (< 97 g/100 g protein) was supplied by Sports Supplements Ltd. (Colchester, UK). Microbial transglutaminase Activa MP from *Streptomyces mobaraensis* was supplied by Ajinomoto Foods Deutschland GmbH, Hamburg, Germany. The activity of the enzyme preparation, which was measured by using the Folk & Cole (1966) method, was 90 units per g.

2.2. Preparation of substrates

Tiger nut milk (TNM) was prepared by wet-milling of soaked and washed tiger nuts using a cutting mill and filter pressing of the mush (Kizzie-Hayford *et al*., 2016). Concentrated TNM (~30 g/100 g total solids), that was obtained after mush separation and evaporation in an R-124 rotational evaporator coupled to a B-172 vacuum controller (BÜCHI Labortechnik AG, Flawil, Switzerland) at 70 °C, was diluted to 10 g/100 g total solids and used as the reference fermentation substrate.

Dispersions of sodium caseinate (8 g/100 g), whey protein isolate (8 g/100 g) and xanthan gum (1 g/100 g) was separately prepared by dispensing the necessary amount in aqua demin. and mixing with a magnetic stirrer at 25 °C for at least 2 h. When applicable, protein

solutions were heated for protein denaturation in a water bath at 80 °C for 10 min, cooled to room temperature and divided into two parts. One part was treated with mTGase according to Jaros *et. al*. (2014a, 2014b): after thermal equilibration of the protein solution and addition of 3 U mTGase per g milk protein, incubation was carried out in a water bath at 40 °C for 2 h. Subsequently, the mixture was heated to 80 °C for 10 min for enzyme inactivation, and immediately cooled in ice water. To prevent effects due to this heat treatment, the protein solution without enzyme treatment was also subjected to all heating and cooling steps. Subsequently, substrates for the fermentation of protein-enriched TNM systems were prepared by mixing TNM with xanthan gum to result in 10.0 g tiger nut solids, 0.1 g xanthan and 3.0 g sodium caseinate or 3.0 g whey protein isolate without mTGase treatment (CnX, WPX) or with mTGase treatment (CnXe, WPXe) per 100 g substrate.

2.3. Fermentation of tiger nut milk substrates

After enrichment, TNM was pasteurized at 70 °C for 15 min in 500 mL plastic jars under continuous agitation, cooled and inoculated with 0.01 g/100 g FVV-211 yogurt starter, a mixed culture of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* (DSM Food Specialties, Delft, Netherlands), and fermented by placing samples in a water bath at 38 °C for 16.5 h. During fermentation, pH was continuously monitored using an InoLab 730 pH meter (WTW GmbH, Weilheim, Germany), and lag time λ (h) and maximum rate of pH reduction μ (1/h) were estimated from pH/time plots using the Gompertz model as described previously (Kizzie-Hayford *et al*., 2016). After acidification, semi-solid TNM gels were homogenized at 11,000 rpm for 20 s using a T25 ultra turrax (IKA GmbH & CO. KG, Staufen, Germany) to ensure smooth texture products. Samples were filled into 120 mL sterile plastic jars and firmly sealed with lids

for 24 h. Subsequently, TNM products were analyzed after 0, 5, 10 and 15 d of storage at 6 °C. Fermentation of TNM products was performed in triplicate.

2.4. Analysis of protein cross-linking and of the fermented tiger nut milk products

2.4.1. Size exclusion chromatography of enzymatically cross-linked proteins

To assess the extent of mTGase cross-linking, protein analysis was performed by size exclusion chromatography (AZURA Assistant ASM 2.1L, Knauer Wissenschaftliche Gerate GmbH, Berlin, Germany) with a UVD 2.1S detector at 280 nm (Knauer Wissenschaftliche Gerate GmbH, Berlin, Germany). The elution buffer, composed of 1 g/L CHAPS, 6 mol/L Urea, 0.1 mol/L NaCl, and 0.1 mol/L Na_2HPO_4,was adjusted to pH 6.8. For dissociating protein aggregates and reducing disulphide bonds, protein solutions were diluted with elution buffer and treated with dithiothreitol of a concentration of 0.15 g/L. Samples were separated and detected by 0.5 mL/min isocratic elution using a Superdex 200 increase 10/30 column (GE Healthcare, Uppsala, Sweden) at ambient temperature. Chromatographic data was acquired using the ClarityChrom v.3.07 software (Knauer Wissenschaftliche Gerate Gmbh) and corresponding peak areas (A) were analyzed for the fractions of monomers, dimers and polymers. Degree of polymerisation (DP, %) was calculated according to Bönisch, Lauber and Kulozik (2004) by DP = 100 Σ (Area[dimers+trimers+polymers])/Σ (Area[monomers+dimers+trimers+polymers])

2.4.2. Viable counts

Viable counts of *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus* in the fermented products were determined by pour plating of the samples diluted in peptone water using MRS or M-17 media, respectively (IDF, 2003). Determinations were done in triplicate.

2.4.3. pH and titratable acidity

pH of the fermented products was measured at 20 ± 1 °C. Titratable acidity was determined according to a previously described procedure (Kizzie-Hayford *et al*., 2016). The average titre of NaOH for each analyte was recorded, and the lactic acid equivalent was calculated according to Sadler & Murphy (2014). pH and titratable acidity (TA) were determined in triplicate.

2.4.4. Syneresis and viscosity

Syneresis of fermented TNM under accelerated gravity was determined as described by Jaros *et al*. (2007) with modifications. 15.0 g fermented product was transferred into pre-weighed tubes. The samples were centrifuged at 1,400 g, 4 °C for 20 min. Subsequently, the separated liquid was removed using a Pasteur pipette. Syneresis was expressed as the relative amount of removed liquid, related to the initial 15 g subjected to centrifugation. Apparent viscosity of fermented TNM was measured using a Physica MCR 301 rheometer (Anton Paar GmbH, Graz, Austria). After storage at 6 °C for 24 h, samples were transferred into a cylinder geometry (inner diameter, 24.66 mm; outer diameter, 26.66 mm; height, 40 mm) and equilibrated to 20 ° C for 5 min before applying a shear rate sweep from 0.01/s to 100/s (Kizzie-Hayford *et al*. 2016). Measurement of syneresis and viscosity were carried out in triplicate.

154 *2.4.5. Colour*

155 The colour attributes of the fermented product were analyzed using a LUCI 100 CIE-Lab
156 colour space colorimeter (Hach Lange GmbH, Düsseldorf, Germany) working with D65 xenon
157 illumination and the 10° standard observer. The instrument was calibrated against black and
158 white standard surfaces (LZM128). Mean values of lightness L^*, red-green intensity a^* and
159 yellow-blue intensity b^* were derived from the colour primaries. The Chroma $C^* = [(a^{*2}) +$
160 $(b^{*2})]^{1/2}$ and the hue angle $h_{ab} = \arctan(b^*/a^*)$ were additionally computed (Rohm & Jaros,
161 1996). Triplicate determinations were made.

162

163 *2.5 Statistical analysis*

164 Data were evaluated using one-way analysis of variance. Tukey HSD or Games-Howell
165 *post hoc* analysis was used to compare the mean values when necessary. SPSS software package
166 version 16.0 was used for performing the analysis (SPSS Inc., Chicago, IL, USA). All
167 significance statements refer to $P < 0.05$.

168

169 ## 3. Results and discussion

170 *3.1. Effect of enrichments on microbial acidification of tiger nut milk*

171 Microbial acidification of plain TNM and TNM enriched with mTGase treated or untreated
172 proteins resulted in pH profiles (Fig. 1) similar to those in our previous report (Kizzie-Hayford *et*
173 *al*., 2016). For plain TNM (dry matter, 10.20 ± 0.4 g/100 g; protein content, 0.89 ± 0.02 g/100 g),
174 the initial pH, $pH_0 = 6.35 \pm 0.09$, and the Gompertz maximum rate μ of pH reduction, $\mu = 0.65 \pm$
175 0.11/h, were slightly higher in the present study. This can be partly ascribed to the differences in
176 tiger nut protein content, which varies at different harvest periods (Asante, Oduro, Ellis, &

177 Saalia, 2014), and to its higher content of acidic amino acids than basic amino acids (Aremo,
178 Bamidele, Agere, Ibrahim, & Aremu, 2015), that influences pH during fermentation of TNM
179 (Kizzie-Hayford et al. 2016). Fermentation of TNM enriched with xanthan and untreated casein
180 (CnX) or whey protein (WPX) resulted in Gompertz equation lag times λ of $\lambda = 1.58 \pm 0.07$ h
181 and $\lambda = 1.24 \pm 0.21$ h, and $\mu = 0.69 \pm 0.04$/h and $\mu = 0.79 \pm 0.07$/h, respectively. Microbial lag
182 times for TNM enriched with cross-linked proteins significantly decreased to $\lambda = 0.95 \pm 0.18$ h
183 (CnXe) and $\lambda \sim 0.66 \pm 0.09$ h (WPXe), respectively, whilst the rate of maximum pH reduction
184 increased to $\mu = 0.79 \pm 0.02$/h (CnXe) and $\mu = 0.91 \pm 0.01$/h (WPXe) (Fig.1). Neve, Lorenzen,
185 Mautner, Schlimme, & Heller (2001) reported that mTGase treatment promotes the initial growth
186 of *S. thermophilus* during milk fermentation. Mixed cultures of *S. thermophilus* and *L.*
187 *delbrueckii* ssp. *bulgaricus* are known to show protocooperation, where *S. thermophilus* with its
188 little or no proteolytic activity initiates fermentation until pH ~ 5.7, and produces formate,
189 pyruvate, folate, CO_2 and long chain fatty acids. These metabolites stimulate the growth of *L.*
190 *delbrueckii* ssp. *bulgaricus*, which generates oligopeptides and amino acids that in turn stimulate
191 the growth of *S. thermophilus* (Baglio, 2014; Hill & Kethireddipalli, 2013). mTGase treatment of
192 milk resulted in larger molecular weight protein polymers (Fig. 2), which are reported to be
193 covalently cross-linked (Jaros *et al*., 2006). After treatment of whole milk with mTGase,
194 fermentation time was reported to be prolonged when using LAB (Lorenzen *et al*. 2002). This
195 was attributed to a decrease in the growth of lactobacilli, assumed to be caused by a limitation in
196 accessible low molecular weight peptides because of the protein cross-linking (Faergemand,
197 Jörgensen, Budolfsen, & Qvist, 1999). Even though λ decreased and μ increased in products
198 enriched with mTGase treated casein in the present study, a significantly longer fermentation
199 time (9.8 ± 0.1 h) was required to reach pH 4.5 than the corresponding untreated systems (CnX =

8.8 ± 0.1 h). On the other hand, to reach pH 4.5, products enriched with WPXe required a fermentation time of 7.2 ± 0.1 h, which was not significantly different from that of the untreated counterpart (WPX =7.3 ± 0.3 h). The effect of mTGase cross-linking of proteins on the time to reach a specific pH during lactic acid fermentation of milk might be related to the nature of proteins (Bönisch *et al*., 2004). After 15 h fermentation, the pH of plain TNM, WPX and CnX systems decreased to pH ~ 4.27 ± 0.05, 4.30 ± 0.03 and 4.36 ± 0.03, respectively. Even though the pH of the fermented products with mTGase treated proteins was not significantly different from the untreated counterparts, they showed a trend of slightly higher pH.

3.2. Effects of enrichment on storage properties

3.2.1. Microbiological effects

All the TNM systems allowed the development of lactic acid bacteria to various extents. The range of viable counts of starter culture after homogenization and storage of products at 6 °C for 1 d were, for *S. thermophilus*, 1.6-5.8 x 10^8 cfu/g, and were higher than those of *L. delbrueckii* ssp. *bulgaricus* (1.1 – 2.2 x 10^6 cfu/g). The viable cell count was lower in the present study, notably for *L. delbrueckii* ssp. *bulgaricus* than our previously reported values (Kizzie-Hayford *et al.*, 2016). Viable counts of *S. thermophilus* from enriched systems containing CnXe or WPXe were slightly but insignificantly (~ 0.04 ± 0.01 log cfu/g and 0.08 ± 0.02 log cfu/g, respectively) higher than those of their untreated counterparts after 0 d storage. In contrast, incorporation of mTGase treated proteins in the TNM systems considerably decreased the viable count of *L. delbrueckii* ssp. *bulgaricus* by approximately 0.30 ± 0.03 log cfu/g compared to the untreated counterparts. This suggests that, enrichment of TNM with mTGase cross-linked proteins might

promote the proliferation of *S. thermophilus* but decrease the growth of L. *delbrueckii* ssp. *bulgaricus*, which might lead to a reduction in post acidification (Xu *et al*., 2015).

The effects of enrichment and storage of fermented tiger nut milk on the viable cell count of lactic acid bacteria during **15 d** is shown in **Fig. 3**. Generally, *L. delbrueckii* ssp. *bulgaricus* showed a more drastic reduction (**Fig. 3a**) than *S. thermophilus*, which exhibited an insignificant decline in viable cell count in all the fermented systems (**Fig. 3b**). The higher decrease of *L. delbrueckii* ssp. *bulgaricus* than *S. thermophilus* is similar to the report by Neve *et al*. (2001) who, however, used milk systems as fermentation substrates. Enrichment of TNM with untreated proteins resulted in a higher rate of reduction of the viable cell counts of *L. delbrueckii* ssp. *bulgaricus* compared to the plain TNM during **15 d** storage. However, it is clear from **Fig. 3a** that the pre-treatment of proteins with mTGase was able to reduce the rate of decline in the viable cell count of *L. delbrueckii* ssp. *bulgaricus* significantly compared to the untreated proteins. mTGase cross-linking might reduce protein accessibility for *L. delbrueckii* ssp. *bulgaricus*, leading to weaker growth and less produced lactic acid during storage. Thus, the inclusion of cross-linked proteins might have relevance for maintaining the microbiological quality of fermented TNM systems during storage.

3.2.2. Physico-chemical effects

Storage of fermented plain TNM at the defined conditions neither affected pH nor titratable acidity of the system significantly (Table 1). However, enrichment with proteins significantly decreased the pH, and consequently, increased TA of the fermented system after **15 d**. Addition of mTGase treated proteins did not show any significant effect on the pH or TA compared to that of the fermented system from their untreated counterparts, even though a trend in slight reduction in TA was observed after **15 d** storage.

According to **Fig 4**, lactic acid fermentation of TNM resulted in products with considerably low viscosity (0.02 ± 0.00 Pa.s at a shear rate of 1/s). Enrichment of TNM with sodium caseinate or whey protein and subsequent fermentation led to products with significantly improved viscosity of 0.56 ± 0.01 Pa.s (CnX) and 1.40 ± 0.04 Pa.s (WPX) at 1/s after 0 d storage. The protein enrichments allowed the formation of firm protein gels during fermentation that arrested phase separation and after homogenization, resulted in higher viscosity (Kizzie-Hayford *et al*. 2016). Enrichment of TNM with mTGase treated casein or whey proteins caused the viscosity of their fermented systems to increase significantly by a factor of ~ 16.4 and ~ 3.6 compared to their untreated counterparts, respectively (**Table 1, Fig. 4**). This is ascribed to the increase in protein aggregate size because of the cross-linking effect of mTGase as can be seen in Fig 2. It was observed that the enzyme treatment under the applied conditions resulted in degrees of polymerization of casein and whey protein of approx. 68 % and 32 % compared to the non-cross-linked protein solution of 13 % and 11 %, respectively. Bönisch *et al.* (2004) observed a DP increase from 14 % to 60 % under similar conditions after mTGase cross-linking of ultra-high temperature treated sodium caseinate solution, and de Jong & Koppelman (2002) reported a more effective cross-linking in caseinate systems than in whey protein systems. Additionally, a trend in increase in the viscosity of the protein enriched systems was evident after 15 d (**Table 1**). Increase in viscosity of stirred yogurt during 9 d storage was reported by Jaros *et al*. (2007) among others. Increasing viscosity during storage is related to re-arrangements in the protein network after breaking up the structure in the stirring step. Even though the enrichment of TNM with untreated proteins increased viscosity, the fermented systems showed considerable susceptibility to syneresis during storage (Fig 4, Table 1). Forced syneresis in fermented plain TNM was ~ 86 %, and that of the WPX enriched system was ~ 32 %, being lower than that of

the systems enriched with CnX (39 %). In our previous study (Kizzie-Hayford *et al*. 2016), non-homogenized fermented TNM enriched with proteins showed lower syneresis in case of casein than that of whey protein, pointing on higher shear resistance of whey protein aggregates than that of casein gels in the present study. Products that were enriched with CnXe or WPXe showed a significantly different syneresis, being approximately one-third lower than that of their untreated counterparts (Table 1). This effect might be ascribed to a more elaborate protein network caused by the mTGase treatment and a corresponding decrease in gel pore-size and increase in viscosity (Jaros *et al*., 2007, 2006; Lorenzen *et al*., 2002). A decreasing trend in the rate of syneresis in the protein enriched systems during storage was observed, which was significant after 15 d (Table 1). Probably, the marginal increase in viscosity of the enriched, fermented systems contributed to reduction in syneresis during the storage period.

3.2.3. Colour

The average lightness L^* of fermented TNM was 64.2 ± 0.80. Fermented systems enriched with protein showed significantly higher lightness of L^* = 69.6 ± 1.10 (CnX) and L^* = 66.5 ± 0.40 (WPX). Systems resulting from addition of CnXe or WPXe showed insignificantly lower L^* than their untreated counterparts, which were 67.6 ± 1.63 and 65.7 ± 0.61, respectively. The colour intensity of the fermented systems were C^* = 11.7 ± 1.5 (TNM), 11.5 ±1.2 (CnX), 10.9 ± 1.5 (CnXe), 11.3 ± 1.4 (WPX) and 11.2 ± 1.2 (WPXe), showing that TNM enrichment with proteins or mTGase treated protein did not significantly affect this parameter. The hue angle, h_{ab} of all fermented systems ranged between 1.4° -1.5°. During storage, the chroma and hue of the fermented systems did not show any significant differences. However, fermented TNM showed the highest and significant lightness decrease, with L^* being ~ 3.8 units lower after 15 d storage

(**Fig. 5**). Formation of Maillard products caused by wet milling of tiger nuts partly contributes to TNM lightness decrease, which increases during storage (Kizzie-Hayford *et al.*, 2015). Recently, peroxidase activity was reported in tiger nut milk (Codina-Torrella, Guamis, Ferragut, & Trujillo, In print). This enzyme is known to catalyze the oxidation of phenolic compounds that are present in tiger nuts (Oladele, Osundahunsi, & Adebowale, 2009) to brown quinone products, which contribute to lightness decrease in TNM and other vegetable milk-like extracts (Queiroz, Mendes Lopes, Fialho, & Valente-Mesquita, 2008). Enrichment with proteins was effective for minimizing decrease in lightness, and whey proteins were more effective than caseins during storage (Fig.5). Improvement in the lightness of protein-enriched fermented TNM might be attributed to the colour-imparting effects of the protein powders. The system resulting from CnXe showed a slightly lower lightness decrease than the untreated counterpart, whilst no clear effect was observed for the WPXe system. Cross-linking of sodium caseinate is known to enhance the stability of the protein against oxidative products (Ma et al., 2012), which explains in part, the minimizing effect on lightness decrease of enriched, fermented TNM.

4. Conclusions

Fermentation of TNM enriched with mTGase cross-linked proteins led to products with a less decrease of lactic acid bacteria compared to that of their untreated counterpart during storage. The effect that mTGase cross-linked proteins show on the time required for the fermented system to reach a specific pH is dependent on the type of protein. Fermentation of TNM enriched with mTGase treated proteins resulted in products with higher viscosity and lower syneresis than that of their untreated counterparts during storage. Fermentation of TNM enriched with protein led to products with improved lightness, which showed less decrease when mTGase treated casein was

314 used for the enrichment compared to that of the untreated counterpart during storage. Thus,
315 incorporation of mTGase treated protein in TNM is promising for improving the microbiological
316 and physico-chemical properties of the fermented product during storage.
317

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323

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412

FIGURE LEGENDS

Fig. 1 Acidification profiles during fermentation of plain (TNM) or enriched tiger nut milk. ◈, plain tiger nut milk;○,● , TNM enriched with 3 (3 mTGase-treated) g/100 g sodium caseinate and 0.1 g/100 g xanthan;□, ■, TNM enriched with 3 (3 mTGase-treated) g/100 g whey protein isolate and 0.1 g/100 g xanthan. Only selected points from continuous average of triplicate pH measurements are displayed.

Fig. 2 Size exclusion chromatogram of microbial transglutaminase cross-linked (a) sodium caseinate or (b), whey protein isolate using 3U mTGase/g protein at 40 °C for 2 h. Full lines, protein without mTGase treatment; broken lines, protein after mTGase treatment.

Fig. 3 Effects of enrichment and storage period on the viable cell count of *Lactobacillus delbrueckii* ssp. *bulgaricus* (a) and *Streptococcus thermophilus* (b) in fermented tiger nut milk (n = 3). ◈, plain tiger nut milk; ○ , ●, TNM enriched with 3 (3 mTGase-treated) g/100 g sodium caseinate and 0.1 g/100 g xanthan; □,■ , TNM enriched with 3 (3 mTGase-treated) g/100 g whey protein isolate and 0.1 g/100 g xanthan.

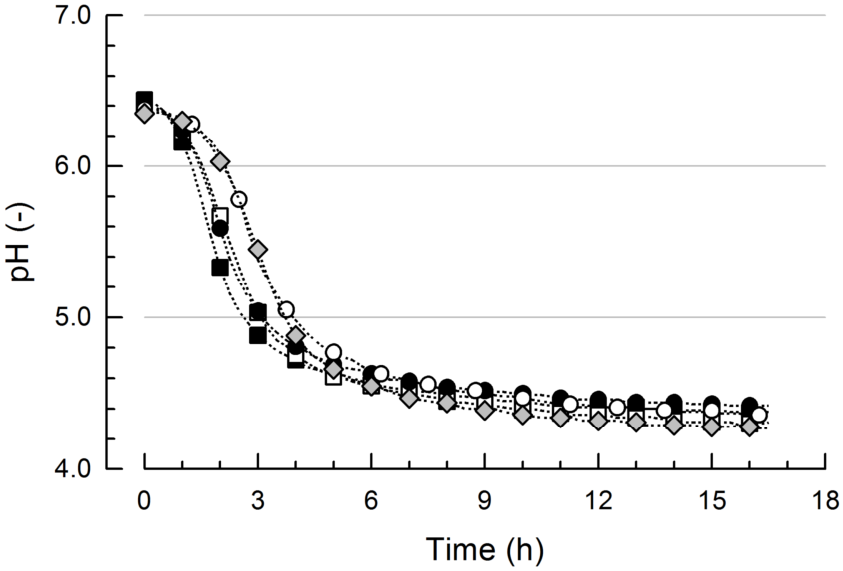
Fig. 4 Apparent viscosity of fermented tiger nut milk (TNM) with different compositions after 1 d storage at 6 °C. ◈ , plain tiger nut milk; ○,●, TNM enriched with 3 (3 mTGase-treated) g/100 g sodium caseinate and 0.1 g/100 g xanthan; □, ■, TNM enriched with 3 (3 mTGase-treated) g/100 g whey protein isolate and 0.1 g/100 g xanthan. Only selected points from continuous triplicate viscosity measurements are displayed.

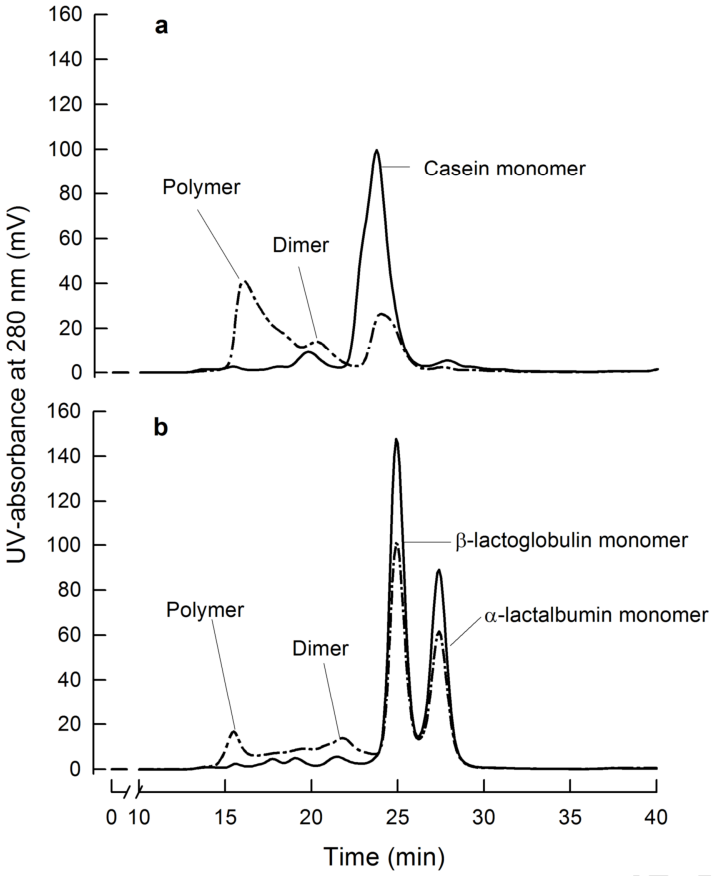
Fig. 5 Effects of protein enrichment and storage period on lightness of fermented tiger nut milk (n = 3). ◈, plain tiger nut milk; ○,●, TNM enriched with 3 (3 mTGase-treated) g/100 g sodium caseinate and 0.1 g/100 g xanthan; □,■ , TNM enriched with 3 (3 mTGase-treated) g/100 g whey protein isolate and 0.1 g/100 g xanthan.

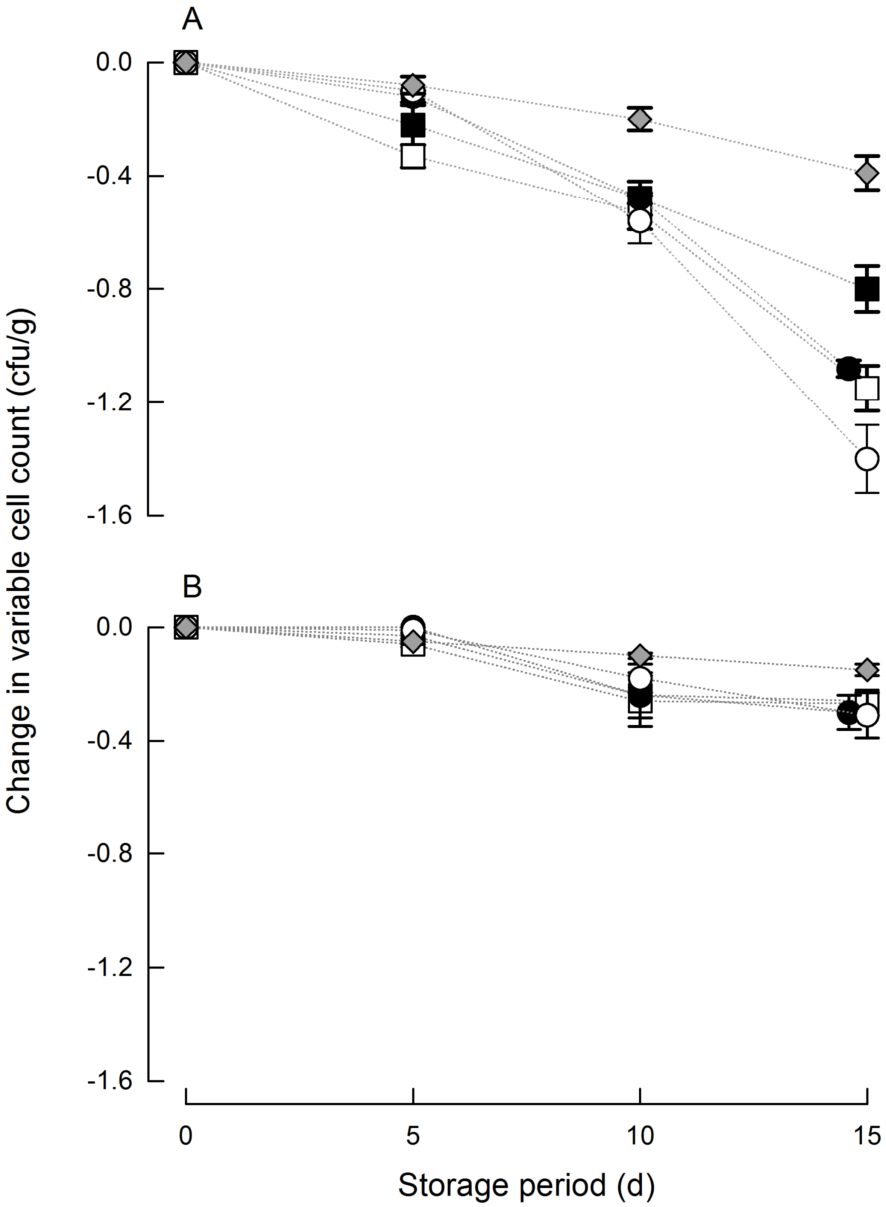
Table 1. Effects of storage period on the physico-chemical properties of fermented tiger nut milk and the enriched systems.

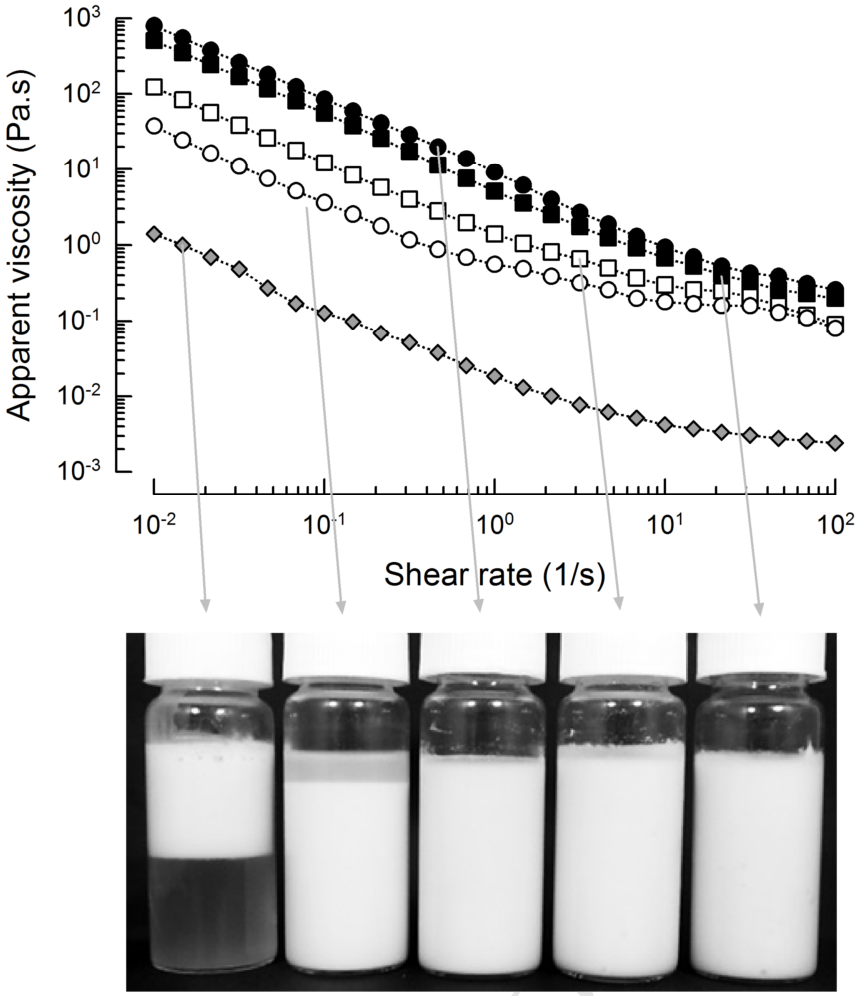
Physico-chemical parameters	Storage (d)	System[a]				
		TNM[b]	CnX	CnXe	WPX	WPXe
pH (-)	0	4.23^{a} ± 0.02	4.27^{a} ± 0.01	4.34^{a} ± 0.02	4.28^{a} ± 0.01	4.31^{a} ± 0.02
	5	4.23^{a} ± 0.01	4.22^{a} ± 0.02	4.29^{a} ± 0.01	4.24^{a} ± 0.02	4.25^{a} ± 0.03
	10	4.22^{a} ± 0.01	4.16^{b} ± 0.01	4.15^{b}± 0.01	4.18^{b} ± 0.02	4.16^{b} ± 0.01
	15	4.21^{a} ± 0.01	4.14^{b} ± 0.03	4.15^{b} ± 0.02	4.16^{b} ± 0.03	4.14^{b} ± 0.03
Titratable acidity (g/100 g)	0	0.52^{a} ± 0.01	1.16^{a} ± 0.07	1.07^{a} ± 0.10	0.93^{a} ± 0.06	0.92^{a} ± 0.13
	5	0.52^{a} ± 0.02	1.24^{ab} ± 0.09	1.17^{ab} ± 0.12	1.00^{ab} ± 0.10	0.97^{a} ± 0.10
	10	0.54^{a} ± 0.01	1.29^{ab} ± 0.11	1.26^{ab} ± 0.10	1.04^{ab} ± 0.08	1.00^{a} ± 0.09
	15	0.54^{a} ± 0.03	1.34^{b} ± 0.08	1.30^{b} ± 0.10	1.07^{b} ± 0.05	1.02^{a} ± 0.09
Viscosity (shear rate, 1.0 1/s (Pa.s))	0	0.02 ± 0.00	0.56^{a} ± 0.01	9.17^{a} ± 1.28	1.40^{a} ± 0.04	5.12^{a} ± 0.16
	5	-	0.61^{a} ± 0.03	9.57^{a} ± 0.54	1.44^{a} ± 0.02	5.14^{a} ± 0.68
	10	-	0.59^{a} ± 0.02	8.93^{a} ± 1.23	1.39^{a} ± 0.07	5.44^{a} ± 1.06
	15	-	0.74^{b} ± 0.08	10.20^{a} ± 0.3	1.47^{a} ± 0.02	6.00^{a} ± 0.86
Syneresis (%)	0	86.2 ± 1.2	38.9^{a} ± 0.1	27.2^{a} ± 0.4	31.5^{a} ± 1.0	20.2^{a} ± 1.6
	5	-	38.0^{b} ± 0.1	24.6^{b} ± 0.3	30.4^{a} ± 1.2	17.9^{ab} ± 2.2
	10	-	36.3^{c} ± 0.4	21.8^{c} ± 0.2	29.8^{a} ± 0.2	17.6^{ab} ± 2.0
	15	-	36.1^{c} ± 0.8	21.5^{c} ± 0.5	27.1^{b} ± 1.2	16.3^{b} ± 0.4

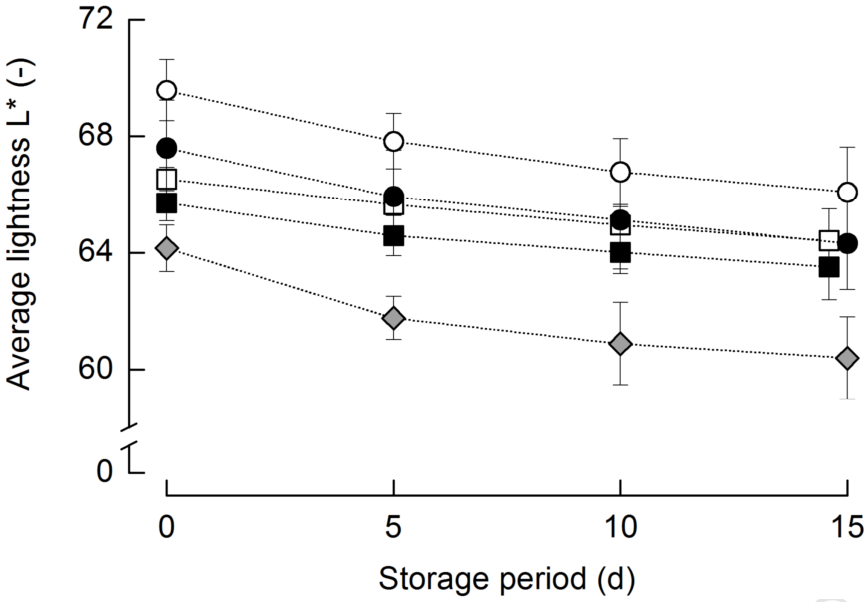
[a]TNM, tiger nut milk; CnX (CnXe), TNM enriched with 3 (3 mTGase-treated) g/100 g sodium caseinate and 0.1 g/100 g xanthan; WPX (WPXe), TNM enriched with 3 (3 mTGase-treated) g/100 g whey protein isolate and 0.1 g/100 g xanthan.
[b]Results are arithmentic mean ± standard deviation from (n=3) determinations. Values in the same column with different superscripts differ significantly at $P < 0.05$.











Highlights:

- Protein cross-linking was investigated for improving fermented tiger nut milk
- Cross-linked sodium caseinate increases fermentation time of tiger nut milk
- Cross-linked proteins minimize starter count decline during product storage
- Cross-linked proteins improve viscosity and syneresis of fermented tiger nut milk
- Protein enrichment improves lightness of fermented tiger nut milk