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Impact of heat treatment and acid gelation on polyphenol enriched milk samples

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ABSTRACT

The effects of heat treatment and the stage of polyphenol addition to milk before or after heat treatment on the total phenolic content (TPC), ferric-ion reducing antioxidant power (FRAP), pH, casein micelle size (CMS) and whey protein denaturation content of milk-polyphenol mixtures were investigated. Four sources of phenolic compounds (green tea, white grape, tannic acid, gallic acid) were incorporated into pasteurized-skim milk. A heat treatment (85 °C for 30 min) was applied to pasteurized-skim milk either before (M_hP) or after polyphenols addition (M_hP_h). Acid milk gels were produced using M_hP_h samples, and their TPC and FRAP were determined. Heat treatment decreased the TPC and FRAP values of the samples, except for tannic acid, probably due to increased polyphenol-protein interactions. However, M_hP_h tannic acid sample resulted in significantly higher FRAP value than M_hP. The addition of gallic acid before heat treatment (M_hP_h) significantly increased CMS due to the lower pH of this sample during heating. Acid gelation decreased the extractable polyphenols, however there was no significant different on FRAP between acid gel and M_hP_h milk samples. This study showed that the properties of phenolic source, particularly pH, and the stage of polyphenol addition to milk had an impact on selected properties.

Key words: polyphenol, milk protein, heat treatment, acid milk gel, antioxidant activity

1. Introduction

Polyphenols are secondary plant metabolites and there are over 8000 known phenolic structures which consist of a hydroxyl group linked to an aromatic ring (Tresserra-Rimbau, Lamuela-Raventos, & Moreno, 2018). Polyphenols are classified according to their carbon skeleton: phenolic acids, flavonoids, stilbenes and lignans (Tresserra-Rimbau, Lamuela-Raventos, & Moreno, 2018). In recent years, there has been increased interest in the study of polyphenols due to their possible health benefits. Previous studies have suggested that they have strong antioxidant capacities, decrease the risk of cancers and cardiovascular disease (Costa et al., 2017).

Milk proteins are natural delivery vehicles of bioactives due to their physicochemical and functional properties (Tavares, Croguennec, Carvalho, & Bouhallab, 2014). Therefore, polyphenols could be delivered by dairy products and the phenolic content and antioxidant activity of the final product might be improved. However, polyphenols may lead to changes in the structural and functional properties of milk proteins as polyphenols interact with milk proteins *via* hydrogen bonding, hydrophobic interactions and covalent bonds, leading to the formation of soluble and insoluble complexes (Jakobek, 2015). These interactions depend on pH, temperature, type and structure of both proteins and polyphenols (Bandyopadhyay, Ghosh, & Ghosh, 2012).

The enrichment of various dairy products with polyphenols has been widely studied in recent years (Aliakbarian et al., 2014; Szwajgier & Gustaw, 2015). Past studies reported contradictory results related to the effect of milk on the antioxidant activity of polyphenols (Keogh, McInerney, & Clifton, 2007; Korir, Wachira, Wanyoko, Ngure, & Khalid, 2014) which is possibly a consequence of either various methods used for the measurement of antioxidant activity, or the phenolic types utilised. Therefore, in this study a phenolic acid (gallic acid), tannin (tannic acid) and polyphenol rich extracts from green tea and white grape were used to understand the effect of phenolic types on the antioxidant activity and acid milk gel product. There is still a lack of information regarding the effect of dairy processing conditions on polyphenols when incorporated in milk. Acid milk gel products (e.g. yogurt) are widely consumed. Thermalization of milk (85 °C for 30 min) is one of the critical steps in the manufacture of this products as it is required to deliver a product of suitable textural quality and water holding capacity (Harbourne, Jacquier & O'Riordan, 2011). Therefore, it is necessary to understand the impact of this thermal process on the polyphenol content, antioxidant activity and stability of proteins in polyphenol enriched dairy products. Furthermore, these properties may also be affected by polyphenol addition before or after the thermal treatment. The objective of the present study was to determine the impact of polyphenol addition, of various types, before or after heat treatment on the total phenolic content, antioxidant activity, casein micelle size & whey protein denaturation of milk and acid gel samples enriched with polyphenols.

2. Materials and Methods

2.1. Experimental Design

Polyphenol solutions were prepared by dissolving polyphenol powders in distilled water (1 mg/mL) without (P) and with heat treatment (P_h) at 85 °C for 30 min to understand the impact of heat treatment on the reconstituted polyphenol powders. To investigate the effect of heating on milk-polyphenol samples a fully randomised experiment was carried out in triplicate based on a 4 x 3 factorial design. 4 sources of polyphenols: green tea, white grape, tannic and gallic acids and 3 sample preparations: pasteurized skim milk-polyphenol mixtures (MP), pasteurized skim milk heated before polyphenols addition (M_hP) and pasteurized skim milk heated after polyphenols addition (M_hP_h) (Table 1). For each treatment, a control sample which has no polyphenols was prepared by adding distilled water in place of the polyphenols.

Acid milk gels were prepared with M_hP_h to determine the effect of acid gelation on the milk-polyphenol mixtures.

2.2. Materials

Dried extracts: green tea (Nutraceutica, Monterenzio, BO, Italy) and white grape (Nutripy CHR-Hansen, Hørlshom, Denmark) and single phenolic compounds: tannic and gallic acids (Sigma Aldrich, Gillingham, UK) were employed in this study. Their phenolic composition is described in Table 2. Pasteurized skim milk was purchased from a local retailer and composition of milk (0.08 ± 0.01 g fat/100 mL, 3.36 ± 0.02 g protein /100 mL, 8.07 ± 0.04 g total solids /100 mL, 4.75 ± 0.06 g lactose/100 mL) was determined by LactoScope FilterAuto (QuadraChem Laboratories Ltd, Forest Row, UK). Sodium carbonate (Na_2CO_3) was supplied by Thermo Fisher Scientific Ltd (Loughborough, UK). Gluconodelta lactone (GDL), hydrochloric acid (HCI, 12 mol/L), methanol, Folin-Ciocalteu reagent, sodium acetate trihydrate, acetic acid, 2, 4, 6-Tris (2-pyridyl)-s-triazine (TPTZ), ferric chloride hexahydrate, ascorbic acid, sodium chloride (NaCI) and all the polyphenol standards that used to quantify green tea and white grape were from Sigma Aldrich (Gillingham, UK).

2.2.1 Individual Phenolic Detection of Green Tea and White Grape

The green tea solution (5 mg/mL) was analysed with Dionex HPLC (Germering, Germany) equipment that contains P680 HPLC pump, ASI-100 automated sample injector, thermostatted column compartment TCC100, PDA-100 photodiode array detector with a Zorbax eclipse XDB-C18 column (4.6 m × 150 mm, 5 µm, 25 ˚C, Agilent). Separation was carried out by a gradient elution using formic acid/water (0.1: 99.9, v/v) (mobile phase A) and formic acid/acetonitrile (0.1: 99.9, v/v) (mobile phase B) with elution scheme as follows: 0-5 min 4% B; 5-40 min from 4% to 25% B; 40-55 min from 25% to 50% B, 55-60 min 50% B. The protocol used a 1 mL/min flow rate and a 50 µL injection volume. Chromatograms were recorded at 280 nm. Identification was based on retention times by comparison with HPLC grade standards and quantification of green tea solution was performed using calibration curves of epigallocatechin, catechin, epicatechin, epigallocatechin-gallate, epicatechin-gallate, gallic acid.

The white grape solution (5 mg/mL) was analysed with a Waters UPLC-MS and Quattro Ultima mass spectrometer (Waters, Manchester, UK) with a C-18 guard column (1.7µ / 50 x 2.1 mm, Kinetex, C18 column, Phenomenex, Macclesfield, UK) was used for the analyses. Separation was carried out by a gradient elution using formic acid/water (0.1: 99.9, v/v) (mobile phase A) and formic acid/acetonitrile (0.1: 99.9, v/v) (mobile phase B) with elution scheme as follows: B was increased from 7% to 75% (0.2 min to 8.3 min), B was decreased from 75% to 7% (9.3 min to 10 min), then the column was equilibrated for 5 min at initial condition (7% B). The total run time was 15 min with flow rate of 0.1 mL/min and injection volume of 10µL. The oven temperature was set at 35°C. Detection was performed using retention time and multiple reaction monitoring transition using positive ion mode (3.35 kV). The quantification of white grape solution was estimated based on the area of 10 µM of each polyphenol standard: resveratrol, quercetin-3-O-glucoside, catechin, epicatechin, epigallocatechin-gallate, procyanidin B2.

2.3. Preparation of Polyphenols in Pasteurized Skim Milk

The stock polyphenol solutions (5 mg/mL) were freshly prepared by dissolving polyphenol powders in distilled water before each experiment. They were added to pasteurized skim milk (MP) and stirred for 30 min at room temperature. The final concentration of polyphenol powders in milk samples was 1 mg/mL. A thermalization step of 85 °C for 30 min, typically used in acid milk gel manufacture to denature whey proteins and enhance gel texture, was applied to pasteurized skim milk before (M_hP) and after (M_hP_h) polyphenols addition. Samples were placed (5 mL) in a shaking (90 rev/min) water bath (Grant Instrument Ltd, Cambridge, UK). After heating samples were rapidly cooled by immersion in ice-water. After preparation, all MP, M_hP and M_hP_h samples were stored at 4 ºC for 2 h until analysis.

2.4. Preparation of Acid Milk Gels

Gluconodelta lactone (GDL) (1.7 g/100 g sample) was added to M_hP_h samples and stirred for 2 min. The samples were incubated in Sanyo Gallenkamp incubator (Leicestershire, UK) at 30°C for 3 h and 45 min until the pH reached a value of 4.6.

2.5. Measurement and Adjustment of pH of the Milk-Polyphenol Mixtures

The pH of each sample was determined using an Orion 3-star benchtop pH meter (Fisher Scientific Ltd, UK) fitted with a glass combination electrode. On addition of gallic acid to pasteurized skim milk, the pH decreased to 6.4. Therefore, to have a representative control for this sample, pasteurized skim milk was adjusted to 6.4 using HCI (1 mol/L) and stored at 4°C overnight to equilibrate.

2.6. Casein Micelle Size and Undenatured Whey Protein Content of Milk-Polyphenol Mixtures

The average CMS of samples was measured using a Zetasizer 5000 (Malvern Instruments Ltd, Worcestershire, UK) according to Chen, Grandison, & Lewis (2012). Undenatured whey protein nitrogen (WPN) of samples was determined by applying the GEA Niro method (GEA NIRO, 2009)

2.7. Extraction of free Polyphenols from Milk and Acid Milk Gel

The polyphenols were extracted from the milk and acid milk gel according to Karaaslan, Ozden, Vardin, & Turkoglu (2011). Briefly, an aliquot of the milk-polyphenol mixture (5 mL) or acid milk gel (10 g) was centrifuged (Sorvall RC 6) at 25,860 x g for 15 min at 20 $^{\circ}$C with 10 mL (milk) or 15 mL (gel) of acidified methanol (methanol containing 100 µL and 150 µL conc. HCI for milk and gel, respectively). TPC and FRAP analysis was carried out on the supernatants. For each replicate, the extracts of milk-polyphenol mixtures were freshly prepared and were used the same day for chemical analyses. The same treatment was applied to pasteurized skim milk without polyphenols for comparison.

2.8. Chemical Analyses

2.8.1. Total Phenolic Content

The total phenolic content (TPC) was determined according to Folin Ciocalteu method as described by (Singleton, 1985), using gallic acid as the standard. The results were expressed as milligrams of gallic acid equivalents (GAE) per millilitre of sample (mg GAE/mL).

2.8.2. FRAP (ferric ion reducing antioxidant power) Assay

The FRAP assay was performed to determine the antioxidant activity (AA) of samples according to Benzie and Strain (1996). An ascorbic acid calibration curve was prepared (R^2 = 0.99). The results were expressed as ascorbic acid equivalents (μmol AAE) per millilitre of sample (μmol AAE/mL sample).

2.9. Statistical Analysis

Results in the text are given as mean values ± standard error (SE). The normality of data distribution was analysed by Kolmogorov-Smirnov test. A one-way analysis of variance (ANOVA) and Tukey's pairwise comparisons were used to identify significant differences between the means of treatment methods and samples. An independent t-test was used for comparison of two means. Results with $p < 0.05$ were considered significantly different. Analyses were performed using SPSS Software for Windows (Version 21.0, Armonk, NY: IBM Corp., USA).

3. Results and Discussions

3.1. Selected Properties of Polyphenol Powders

The dominant compounds in green tea were epigallocatechin-gallate (413.99 ± 14.18 mg/g powder) and catechin (373.77 ± 9.24 mg/g powder) (Table 2), this is in agreement with previous studies (Jaziri, Ben Slama, Mhadhbi, Urdaci, & Hamdi, 2009). The phenolic compounds analysed in white grape (Table 2) were also in agreement with previous studies (Wittenauer, Maeckle, Sussmann, Schweiggert-Weisz, & Carle, 2015).

Gallic acid had the highest TPC among the samples, followed by tannic acid, green tea and white grape in descending order (Table 3). There was no significant difference between the TPC of tannic acid and green tea. Similarly, the TPC of green tea and white grape are not significantly different. However, when the concentration of individual phenolic compounds were detected via HPLC and LC-MS, the concentration of the sum of all phenolic compounds in green tea was nearly 8 times more than the concentration of the sum of all phenolic compounds in white grape (Table 2). This indicated that there are some other phenolic compounds in white grape that contributed the TPC, whereas those phenolics were not quantified. The polyphenol powders used in this study have a much higher TPC than regular teas, vegetables and fruits (Gharras, 2009; Dubeau, Samson, & Tajmir-Riahi, 2010). This is possibly due to dehydrated form of polyphenol powders used. The levels of TPC in the polyphenol powders that used in the present study are within the expected range as compared to the commercial grape extracts in the study of da Silva, Matumoto-Pintro, Bazinet, Couillard, and Britten (2015). The pH of green tea, white grape, tannic acid and gallic acid solutions were 6.71, 6.79, 6.40, 3.62 respectively (Table 4).

The FRAP of polyphenol powders correlated (r: 0.796, p: 0.01) with the total phenolic content results, gallic acid had the highest FRAP and it was followed by tannic acid, green tea and white grape (Table 3). The FRAP value of green tea and tannic acid were not significantly different.

3.2. pH of Polyphenol Enriched Skim Milk

Table 4 demonstrates that, as expected (Fox, 1981), the application of a heat treatment (M_hP and M_hP_h) reduced the pH of all samples as compared to MP samples. The pH of the samples containing gallic acid was significantly lower than all the other samples studied. This is due to the acidic properties of gallic acid (pKa 4.41). The stage of polyphenols addition to milk had no impact on pH (M_hP versus M_hP_h).

3.3. Effect of Heat Treatment on Polyphenol Enriched Skim Milk

3.3.1. Total Phenolic Content and FRAP

Before examining the impact of heat treatment on the polyphenol enriched milks the effect of heat treatment (85°C for 30 min) on TPC and FRAP of the polyphenol powders reconstituted in water (1 mg/mL) was determined. Heating the polyphenol solutions had no significant effect on the FRAP of the solutions. Furthermore, there were no significant differences between the TPC of green tea and white grape solutions after heating. However, the TPC of both tannic and gallic acid solutions significantly decreased by 16% and 7% respectively after heating ($p = 0.038$, $p = 0.033$) (Table 5). This suggests that multiple phenolic compounds present in a solution may combine to have a protective effect in comparison with solutions with individual compounds. This is supported by the results of previous studies (Sari, Wijaya, Sajuthi, & Supratman, 2012; Volf, Ignat, Neamtu, & Popa, 2014).

Fig. 1A represents the TPC of samples subjected to MP, M_hP and M_hP_h treatments. Adding polyphenols significantly increased the TPC of all the samples as expected. The TPC of control pasteurized skim milk is not affected by heat treatment. Pasteurized skim milk heated before polyphenols addition (M_hP) resulted in a significant decrease in the TPC of milk containing tea, grape and tannic acid as compared to MP samples. This is probably due to increased interactions between milk proteins and polyphenols (Arts et al., 2002; Wu et al., 2013). It was previously observed that the binding of epigallocatechin gallate (EGCG) was higher with preheated beta lactoglobulin at 75-85 ºC for 20 min as compared to native protein at room temperature (Shpigelman, Israeli, & Livney, 2010). The binding interaction between EGCG and beta lactoglobulin was attributed to hydrophobic interactions and hydrogen bonding. Heating results in denaturation of whey proteins revealing hydrophobic and sulphur containing groups (Taterka & Castillo, 2015); which increases the probability that polyphenols will bind to the protein. It has also been shown that heat-induced denatured whey proteins played a role in strengthening casein-polyphenol interactions (Yazdi & Corredig, 2012).

With the exception of samples containing tannic acid, a similar decrease in TPC was evident when pasteurized skim milk heated after polyphenols addition (M_hP_h) was compared to MP (Fig. 1A). However, the TPC was only significantly lower for green tea samples. As mentioned above, when the tannic acid solution was heated the TPC decreased by 16%. However, no significant decrease was evident when tannic acid was heated with milk (M_hP_h V MP). This suggests that milk may have a protective effect on the phenolic compound in the sample. This has been previously demonstrated for anthocyanins extracted from corncob (Jing & Giusti, 2005). Overall, heat treatment had a significant effect on the TPC of milk-polyphenol mixtures. The stage of polyphenol addition (M_hP v M_hP_h) had no significant effect on the TPC of milk-polyphenols.

The addition of polyphenols to milk significantly increased the antioxidant activity as measured by FRAP for all samples (Fig. 1B) Additionally, heat treatment had no significant effect on FRAP of control sample. Heat treatment, regardless of stage of polyphenol addition, either decreased or had no impact on the FRAP of the samples. These results are inline with the TPC results in Fig. 1A, indicating that in general increased interaction between milk proteins and the polyphenols occured. This is most likely due to the interaction between polyphenols and milk proteins which have been denatured, as previously mentioned. The FRAP of tannic acid added to pasteurized skim milk before heat treatment (M_hP_h) was significantly higher than tannic acid added after heat treatment (M_hP). Heat treatment may increase the hydrolysis of tannic acid, producing gallic acid and galloyl groups when tannic acid added to milk before heat treatment (Kim, Silva, & Yung, 2011). Hydrolysed gallic acid and the hydroxyl groups newly formed on the galloyl group as a result of thermal hydrolyses could be responsible for the increased antioxidant activity of tannic acid (Kim, Silva, Kim, & Yung, 2010). The stage of polyphenol addition had no effect on FRAP of any other sample.

Overall, with the exception of tannic acid, while heat treatment reduced the TPC and FRAP of polyphenol enriched samples, polyphenol addition before or after heat treatment did not significantly impact on TPC and FRAP values.

3.3.2. Casein Micelle Size and Undenatured Whey Protein Amount of Polyphenol Enriched Skim Milk

Fig. 1C presents the CMS of MP, M_hP and M_hP_h samples. Regardless of the sample, the CMS increased after heat treatment for samples where polyphenols were added after the milk was heat treated (M_hP), with the exception of gallic acid, as compared to MP samples and these increases were significant. This is in agreement with previous studies that show whey proteins become denatured following heat treatment. The denatured whey proteins, mainly β-LG and α-LA, intreact with κ-casein by forming disulfide bonds on the surface of micelle and this attachment leads to ultimately increasing the micelles average diameter (Dalgleish & Corredig, 2012; Martin, Williams, & Dunstan, 2007). However, when polyphenols were added prior to heat treatment (M_hP_h) in all cases, except gallic acid, the CMS tended to be smaller than for the M_hP sample, albeit this decrease was insignificant for green tea. This is probably because the M_hP_h samples were more dilute during the heat treatment which leads to lower levels of whey protein denaturation and hence smaller CMS. This is supported by the levels of undenatured whey protein that were detected in the samples (Fig. 1D). In general, M_hP_h samples did show higher levels of undenatured whey protein in comparison to M_hP sample, albeit the differences were not statistically significant for control, green tea or white grape. The trend for the gallic samples was different. Specifically, when gallic acid was heated together with the milk (M_hP_h) the CMS was larger than all other samples. This is probably related to the heat treatment of the sample at a lower pH than other samples (Table 4). Heating milk proteins at lower pH values results in a higher attachment of denatured whey proteins to casein micelles (Taterka & Castillo, 2015). To understand the effect of the pH of pasteurized skim milk on the CMS and undenatured whey protein amount, the pH of pasteurized skim milk was adjusted to 6.41 and heated at 85ºC for 30 min. The CMS and undenatured whey level of the pH adjusted sample was not significantly different to the M_hP_h gallic acid sample (Table 6).

3.4. Effect of Acid Gel Formation on TPC and FRAP

The effect of polyphenol addition on the TPC of M_hP_h samples was compared to acid milk gels prepared with M_hP_h (Fig. 2A). As expected the acid milk gels containing polyphenols had a significantly higher TPC than the control gel (Chouchouli et al., 2013; Karaaslan, Ozden, Vardin, & Turkoglu, 2011). There was a significant decrease in the TPC of acid gel samples in comparison to M_hP_h milk samples for all types of polyphenols studied. The decrease in the level of extractable polyphenols in the acid milk gels is probably because they are very tightly bound to the casein.

There was no significant difference in FRAP values between M_hP_h milk and acid milk gel samples, with the exception of acid milk gels containing green tea which had a significantly higher FRAP value than milk containing green tea (Fig. 2B). Overall, there is poor correlation between the total phenolic content and antioxidant activity in polyphenol enriched acid milk gels, which is in agreement with previous studies on yoghurt (Trigueros, Wojdylo, & Sendra, 2014). They attributed this poor correlation to the complex nature of yogurt. It is possible that whey protein and polyphenol complexes in the supernatant exhibited antioxidant activity, which may explain the reduction in TPC without an impact on FRAP. Almajano, Delgado, and Gordon (2007) found that mixing whey proteins (BSA, beta-lactoglobulin, alpha-lactalbumin) with EGCG (antioxidant from green tea) resulted in the formation of a complex with antioxidant activity. The antioxidant of the complex formed increased during 7-d storage at 30 ºC possibly due to increased interactions between the polyphenol and protein. In the present study, it is possible that the acidification conditions may have resulted in increased interactions between the whey proteins in the supernatant and polyphenols resulting in complexes with antioxidant activity (Zhang et al., 2014).

4. Conclusions

This study showed that heating the polyphenol solutions at 85ºC for 30 min had no significant effect on the FRAP of the solutions. However, heat treatment of milk reduced the TPC and FRAP of polyphenols, except for tannic acid, regardless of whether polyphenols were added to milk before (M_hP_h) or after (M_hP) heat treatment. This suggests that thermal denaturation of whey proteins may increase the binding capacity of proteins to polyphenols. The FRAP value of tannic acid was significantly higher when it is added to milk before heat treatment in comparison to addition of after heat treatment. The lower pH of gallic acid resulted in a significant increase in the CMS of milk when the gallic acid was added before heat treatment. This is related to higher attachment of denatured whey proteins to casein micelle due to lower pH of milk with gallic acid addition during heat treatment. Acid gel processing decreased the extractable polyphenols as compared to M_hP_h milk samples, however there was no significant decrease in FRAP of polyphenol enriched milk gels.

The findings of this study demonstrated that commercial polyphenol extracts, and single phenolic compounds could successfully be incorporated into milk used for production of acidified dairy products. However, the acid dissociation constant of a polyphenol and the stage at which it is added to the milk needs to be considered when enriching milk samples as this may impact on selected properties of the samples. The current study can be applied to yogurt and dairy based deserts and the effect of starter culture on the polyphenols under processing conditions could be investigated.

5. Acknowledgments

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Figure Captions

Fig. 1. (A) Total phenolic content (TPC, mg GAE/mL), (B) ferric ion reducing antioxidant power (FRAP, µmol AAE/mL), (C) casein micelle size (d.nm), (D) undenatured whey protein amount (mg N/g) of milk-polyphenol mixtures following three different treatments (■: MP, ▩: M_hP, □: M_hP_h). MP: pasteurized skim milk-polyphenol mixtures, M_hP: pasteurized skim milk heated before polyphenols addition, M_hP_h: pasteurized skim milk heated after polyphenols addition, GAE: gallic acid equivalents, AAE: ascorbic acid equivalents, d.nm: diameter.nanometer, N: nitrogen. Different letters indicate significant differences at $p < 0.05$ among three treatments for each sample. Error bars represent means of three replicates ± the standard errors.

Fig. 2. The effect of heat treatment and acid gelation on (A) total phenolic content (TPC, mg GAE/mL) and (B) ferric ion reducing antioxidant power (FRAP, μmol AAE/mL) of milk-polyphenol mixtures. GAE: gallic acid equivalents, AAE: ascorbic acid equivalents. Different letters indicate significant differences at $p < 0.05$ between ■: M_hP_h milk and □: M_hP_h acid milk gel for each sample. M_hP_h: pasteurized skim milk heated after polyphenols addition. Error bars represent means of three replicates ± the standard errors.