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Effect of Milk and Brewing Method on Black Tea Catechin Bioaccessibility

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ABSTRACT: The aim of this study was to investigate whether milk reduces the bioaccessibility of tea catechins, which would compromise tea beneficial effects ascribed to polyphenols. Adding milk to black tea has been shown to lead to polyphenol−protein complexes. So far, data on the intestinal stability of polyphenol−protein complexes are scarce. English black tea (0.93 ± 0.06 mol/L total catechins) and Indian black tea (1.83 ± 0.08 mol/L catechins) were prepared with skimmed or full-fat milk and subjected to simulated gastric, small intestinal, and brush border digestion. Adding milk (5.6−40%) to tea results in a decrease of total catechin (TCAT) recovery. However, the bioaccessibilities of TCAT of tea with milk versus tea controls were comparable ($p > 0.05$). The type of milk did not influence TCAT recovery during all digestive stages ($p > 0.05$). Polyphenol−protein complexes are degraded during digestion. It is very unlikely that consumption of tea *with* or *without* milk will result in differences in catechin plasma concentration.

KEYWORDS: consumer habits, English black tea, Indian black tea, in vitro digestion, polyphenols, protein, proteolysis

■ INTRODUCTION

Tea (*Camellia sinensis*) is associated with health and is, after water, the most consumed beverage. Many health effects are attributed to tea; a full review on suggested health effects would be beyond the scope of this paper, but some recent papers address this at length.[1−5] Likely, the claimed or proven health effects reside in the most prominent phytochemicals in tea, the tea polyphenols. For tea, they are specified as a subgroup of polyphenols, namely, flavonoids and more specifically the catechins ((+)-catechin, (+)-gallocatechin, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epigallocatechin-3-gallate (EGCG), of which EC, EGC, ECG, and EGCG are most important).[6,7]

Not all teas contain the same amount and distribution of catechins, depending on the kind of tea, for example, green tea (GT) or black tea (BT), and on the brewing protocol, which is culturally determined. About 80% of the tea consumed worldwide is BT but is prepared using different infusion times and amounts of dry weight of tea used, either as leaves or as tea bags. Also, the addition of milk and sugar to tea is consumer preference or culturally determined, but can have an effect on the availability of catechins. In India and England, two countries with large BT consumption, tea is typically consumed with milk or with milk and sugar. It is known that proteins can strongly interact with polyphenols in general[8,9] and also with catechins,[10] leading to a reduced availability of the catechins, which could influence the health effect of tea with milk versus tea without milk. Initial studies on BT−(milk) protein interactions looked into the effect the proteins have on the antioxidant (AOX) properties in vitro, without simulated gastrointestinal digestion, as well as in vivo.[10−15] However, the link that was made between the AOX effects of tea and the likely health effects via the AOX properties is invalidated by the rather small effect tea AOX present in plasma have compared to the plasma or total body AOX capacity itself.[16] More recently, an interesting health effect attributed to tea, but not directly linked to AOX effects, is in the cardiovascular area. BT as well as GT has clear effects on vascular function, indicated by positive effects on flow-mediated dilation (FMD).[17] The tea ingredients responsible for the increase in FMD are still unknown, but may be the flavonoids. Dietary flavonoids may improve endothelium-derived nitric oxide bioactivity, which is mediated by enhanced nitric oxide synthesis or by decreased superoxide-mediated NO breakdown.[18] Flavonoids may increase nitric oxide production by endothelial cells,[19,20] possibly by stimulating enzyme (Akt)-mediated endothelial-derived nitric oxide synthase activity,[21,22] and additionally decrease levels of the vasoconstrictor endothelin-1.[19] Furthermore, methylated flavonoids formed as phase I metabolites inhibit NADPH oxidase activity, thereby reducing the generation of reactive superoxide and hydroxyperoxide.[23]

One study by Lorenz et al., however, claims that the positive effect of tea on FMD is abolished by the addition of milk to tea.[24] The authors suggested that this suppression of vasodilatory effect was due to flavonoids binding to milk proteins, in particular caseins. However, Lorenz et al. measured FMD at a single time point, 2 h after tea consumption, and may have missed the positive effect on FMD. It is known that catechin−casein complexes might remain in the stomach for an extended time.[25] A better understanding of how polyphenol bioavailability is influenced by beverage composition is critical to the development of beverage products to deliver specific health benefits.[26]

To date, only two in vitro studies on catechin−milk protein interaction with respect to catechin availability after simulated digestion are described, which have been performed with only GT and milk.[27,28]

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Table 1. Black Tea Formulations[a]

sample name	tea (% v/v)	water (% v/v)	milk (% v/v)	sugar (% w/v)
EBT + water*	94.4	5.6		
EBT + skimmed milk	94.4		5.6	
skimmed milk blank		94.4	5.6	
EBT + water*	85	15		
EBT + skimmed milk	85		15	
skimmed milk blank		85	15	
IBT + water*	75	25		
IBT + whole milk	75		25	
IBT + semiskimmed milk	75		25	
IBT + semiskimmed milk + sucrose	75		25	4
whole milk blank		75	25	
IBT + water*	60	40		
IBT + whole milk	60		40	
IBT + semiskimmed milk	60		40	
IBT + semiskimmed milk + sucrose	60		40	4
whole milk blank		60	40	

[a] Tea preparation protocols as described under Materials and Methods (English black tea (EBT) concentration = 1 bag per 170 mL water, Indian black tea (IBT) concentration = 20 g leaves/L). Tea controls, tea with extra water to displace milk volume, are marked with an asterisk (*).

We therefore studied in detail the catechin−milk protein interaction as a function of the brewing method (using black tea with English or Indian protocol), milk addition (5.6−40%), type of milk, and addition of sugar via total catechin determination. To understand the impact of proteolytic activity in each digestive step, that is, on this interaction resulting in catechin−protein complexes, catechin concentrations were monitored after each step.

Furthermore, the stability of the catechin−milk protein interaction during simulated digestion was monitored via SDS-PAGE.

MATERIALS AND METHODS

Tea Preparation. English black tea (PG tips one cup tea bags with tags, Unilever U. K. Ltd., United Kingdom) infusions were prepared by the addition of 1 tea bag (containing 2.6 g of leaves) per 170 mL of freshly boiled tap water, infused for 3 min with stirring. The tea bag was squeezed with a spoon and removed before milk or water (to displace milk volume) was added. Samples (3 times 90 mL of each formulation, without filtering) were stored under argon at −20 °C until use.

Four types of English black tea (EBT) formulations and two skimmed milk controls (Table 1) were prepared using the English tea infusion protocol.

Indian black tea (Brooke bond, red label, Hindustan Unilever Ltd., India) brews were prepared as follows: 2 g of tea leaves was added per 100 mL of boiling tap water, and boiling was continued while the needed amount of milk, milk and sugar, or water was added; the brew was boiled for another 5 min. During a cooling period of 10 min, tea brews were filtered through a tea strainer and a paper filter (Merck filter papers 315, 185 mm). Samples (3 times 90 mL of each formulation) were stored under argon at −80 °C until use.

Eight types of Indian black tea (IBT) formulations and two milk controls (Table 1) were prepared using the Indian tea brewing protocol.

The used whole milk (3.5% protein, 3.5% fat), semiskimmed milk (3.5% protein, 1.5% fat), and skimmed milk (3.5% protein, 0% fat) were all from Friesche vlag langlekker (FrieslandCampina, Amersfoort, The Netherlands), and crystal sugar from portion sticks was used (Albron, De Meern, The Netherlands).

In Vitro Simulated Digestion. A three-stage in vitro digestive model mimicking gastric, intestinal, and brush border situations was used for each tea formulation on three different days. The two-stage in vitro digestive model was adapted from Miret et al.[29] using 80 mL of tea formulation and 30 min of incubation with pepsin. For the intestinal phase, the simulated digestion sample was placed in an anaerobic chamber (MACS-VA500-microaerophilic workstation, DW scientific, using 85% N_2, 10% CO_2, and 5% H_2 gases) at 37 °C, and a water solution of anaerobic $NaHCO_3$ (0.1 mol/L) was added together with a mix of pancreatin and bile extract, without use of a dialysis bag. The amount of anaerobic $NaHCO_3$ added was able to adjust the simulated digestion to pH 6.5. The flask was closed with a balloon in the anaerobic chamber and was further incubated for another 2 h in a shaken water bath (Julabo SW22, Julabo labortechnik GmbH, Seelbach/Black Forest, Germany) at 37 °C with continuous mixing (100 rpm). Final pH values after 2 h of intestinal simulation were between 6.7 and 7.9.

A third stage was introduced using brush border membrane (BBM) isolate. BBM isolates were prepared from juvenile porcine intestines as described by Booth et al.[30] and were a gift from Nutreco B.V. (Boxmeer, The Netherlands).

After the intestinal phase, an aliquot (570 μL) was taken, and simulated in vitro BBM digestion was started under anaerobic conditions by addition of BBM isolate (75 μL, containing 1.34 mg/mL protein including enzymes). The samples were incubated in a water bath for 120 min at 37 °C in airtight capped vials (safe-lock, Eppendorf AG, Hamburg, Germany), followed by sample stabilization and storage at −80 °C.

Sample Stabilization. After each digestive stage, an aliquot (4 mL) of brewed tea or digesta was added to a mixture of 0.5 mL of acetonitrile and 0.5 mL of leaf stabilizing solution (2.5 mg/mL EDTA and 2.5 mg/mL ascorbic acid in water) in the above-described anaerobic chamber. Aliquots (0.5 mL) were divided in airtight capped vials and stored at −80 °C until catechin analysis.

For the samples obtained after digestion with BBM isolate, 600 μL of sample was added to a mixture of 75 μL of acetonitrile and 75 μL of leaf stabilizing solution.

All EBT digesta and two IBT brews were not stabilized but directly stored at −20 °C after sampling under anaerobic conditions.

Total Catechin Determination. Recovery of total catechin (TCAT) in the soluble fraction was determined after each digestive stage. The analysis is based on the formation of a catechin-specific colored complex with *p*-dimethylaminocinnamaldehyde (DMACA)[31] and is adapted from Kivits et al.[32]

Digestion samples (from either day 1, 2, or 3) including tea without treatment and milk blanks were assayed completely on the same day in twofold. Samples used in the DMACA assay were centrifuged for 10 min at 15600*g*. The soluble fraction of all samples was diluted in water prior to analysis, giving comparable end dilutions.

Standard solutions (250 μmol/L) of (+)-catechin (97% purity, Unilever, Shanghai, China) were prepared in methanol (Lichrosolv, Merck BV, Schiphol, The Netherlands) and stored under argon at −20 °C. On each measurement day the exact concentration was determined spectrophotometrically (Shimadzu UV1601 UV−vis spectrophotometer) using Beer's law ($E^{1\%}_{1\ cm}$, 280 nm = 141).

TCAT was determined by adding 1 mL of DMACA reagent (1 mg/mL DMACA (Sigma-Aldrich, Zwijndrecht, The Netherlands) in 8:1:1 methanol, water, perchloric acid solution (70%, Riedel-de Haën, Sigma-Aldrich, Zwijndrecht, The Netherlands)) and 10 μL of (+)-catechin standard (5−125 μmol/L in methanol) or sample into a semimicrocuvette. The mixture was thoroughly stirred with a spatula and closed, and at $t = 6$ min an absorption spectrum between 500 and 750 nm was recorded against

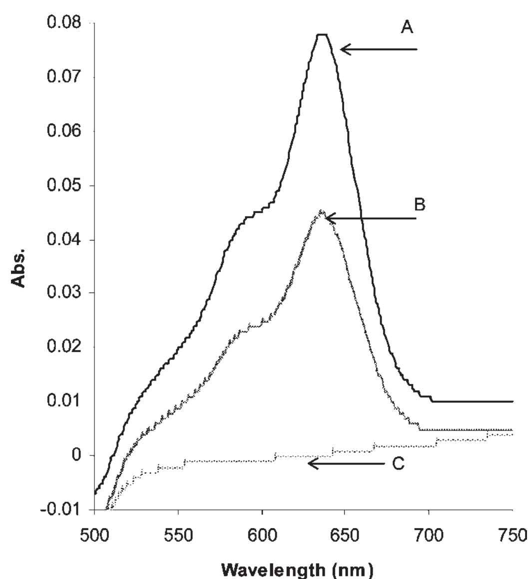

Figure 1. Representative absorption spectrum of the DMACA−catechin reaction product. Spectrum A, IBT (75%) + water (25%), and spectrum B, IBT (75%) + whole milk (25%), are representative for all tea samples. Spectrum C, water (75%) + whole milk (25%), is representative for all milk blanks and shows the absence of any background signal from milk. The samples containing tea all showed peak areas between 1 and 2.5, which lay within the linear area (0.1−3) of the (+)-catechin standard series.

DMACA reagent. The peak area between 603 and 683 nm was used to determine the TCAT content. Results are expressed in micromoles per liter (+)-catechin equivalents. All standard curves, $n = 3$, showed a linear regression correlation coefficient of >0.998.

A representative absorption spectrum of the DMACA−catechin product is shown in Figure 1. The spectrum shows a clear maximum at 637 nm and a shoulder at 590 nm for both tea and tea with milk as described for plasma EDTA samples by Kivits et al.[32] As expected, blank milk digesta showed no response with DMACA (Figure 1). The samples containing tea all showed peak areas between 1 and 2.5, which lay within the linear area (between 0.1 and 3) of the (+)-catechin standard series.

Horizontal SDS Gel Electrophoresis. Excelgel SDS, gradient 8−18%, was used according to the manufacturer's instructions (Amersham Biosciences, 80-1310-00, December 2000), using Coomassie staining. A multiphor II system (GE Healthcare Europe GmbH, Diegum, Belgium) was used to run the gel. Samples, without centrifugation, were diluted to similar end dilutions in tris buffer (0.1 mol/L pH 8.0) and 1:1 mixed with reducing buffer (0.05 mol/L tris, pH 7.5, containing 10 g/L SDS, 1.5 g/L DTT, and 0.1 g/L Bromophenol Blue). Vials were heated for 5 min at 99 °C, and 15 μL of each sample was applied to the gel. Low molecular weight (LMW) marker (GE Healthcare Europe GmbH) was dissolved in 200 μL of reducing buffer and heated for 5 min at 99 °C, and 20 μL was applied in each marker lane.

Statistical Analysis. The data presented are expressed as the mean ± SEM of three or more different experiments. Significant differences were analyzed by one-way ANOVA, and a Newman−Keuls multiple-comparison post test was used for comparisons (Graph Pad Prism 4.01 software, San Diego, CA). Differences were considered to be significant when $p < 0.05$.

RESULTS AND DISCUSSION

Effect of Brewing Method on Catechin Recovery. Following the EBT infusion protocol 0.93 ± 0.06 mol/L ($n = 6$) TCAT was detected in the EBT control brew for 100% EBT without added stabilizer. This is in line with figures described by Kyle et al.,[33] who determined the catechin levels in tea brews (3 g of leaves/400 mL) of six EBT brands using the DMACA method as described by Kivits et al.[32]

Following the IBT brewing protocol, 1.83 ± 0.08 mol/L ($n = 6$) TCAT was detected in 100% IBT brew without stabilization, which is similar to the TCAT level as detected in Brooke bond tea by Reddy et al.,[34] who also used the DMACA method.[32] More catechins were detected in 100% stabilized IBT (5.28 ± 0.23 mol/L ($n = 6$)).

Effect of Milk Amount and Type and Sugar on Catechin Recovery. Addition of milk to either EBT or IBT results in a decrease of TCAT recovery. With low amounts of milk (5.6%) added to EBT, the TCAT recovery was 59 ± 7.2%, whereas for higher amounts of milk (15−40%) added to EBT and IBT about 40% of TCAT remained (Table 2), irrespective of the type of milk. A similar decrease of TCAT recovery after the addition of milk (10−50%) is reported for GT.[27]

Addition of sucrose (40 g/L) to IBT with 25% of semi-skimmed milk seems to prevent catechin−milk protein interaction. In the brewed tea, 1550 μmol/L catechin equivalents is determined in IBT with semiskimmed milk and 2150 μmol/L in IBT with semiskimmed milk and sucrose added (Figure 2A, $p < 0.001$). The stabilizing effect of sucrose is not observed in IBT with 40% milk (Figure 2B).

Effect of Sample Stabilization on Catechin Recovery. The first series of experiments with EBT infusions and some IBT brews were performed without sample stabilization using EDTA and ascorbic acid. We planned to determine the total polyphenol content of the tea digesta using the well-known Folin−Ciocalteu assay,[35] but both ascorbic acid and EDTA interfere with the Folin−Ciocalteu assay.[35,36] It also turned out that amino acids formed in the milk digesta controls during simulated digestion interfered too much with the Folin−Ciocalteu assay, in contrast with intact proteins, which hardly showed any signal (data not shown). The more sensitive and catechin-specific DMACA assay was therefore selected as being more appropriate to follow the experiments.[32]

We found that there was an effect of stabilizer on the original sample and after the gastric phase (Table 2). Stabilized IBT and nonstabilized IBT TCAT recoveries were comparable ($p > 0.05$) after both intestinal and BBM digestion; therefore, we assume that the results of the bioaccessible fractions of stabilized EBT correspond to the data of the nonstabilized EBT. Our in vitro digestion results of EBT correlate well with in vivo findings of van het Hof et al.[37,38] and Kyle et al.,[33] who both measured plasma levels of catechin after ingestion of EBT or EBT with, respectively, 16 and 25% semiskimmed milk and found no significant differences.

When IBT + water without stabilization is compared to IBT + water with stabilization (Figure 2), a remarkable difference in TCAT levels in both the original brewed tea and after gastric phase conditions is observed. Stabilization of the samples with ascorbic acid and EDTA directly after sample taking results in higher TCAT levels than storage under argon without stabilizer. The effect of the stabilizer before digestion is also seen if milk is added to tea. The ratios of stabilized versus nonstabilized samples of the difference in TCAT levels seen before digestion are similar for IBT + water and IBT with whole milk and are independent of the amount of milk added (Figure 2 and Table 2). The effect of the stabilizer is not seen for the intestinal and BBM phases. The digestive recoveries both after gastrointestinal digestion and after

Table 2. TCAT Recovery (Percent) in the Soluble Fraction of BT Formulations before and after in Vitro Digestion (±SEM, $n = 3$)[a]

	tea	G	G&I	G&I&BBM
without stabilization				
94.4% tea + 5.6% water or milk				
EBT + water*	100 ± 8 a	65 ± 5 b	99 ± 5 a	97 ± 13 a
EBT + skimmed milk	59 ± 7 b	54 ± 6 b	74 ± 3 bc	84 ± 15 ac
85% tea + 15% water or milk				
EBT + water*	100 ± 6 a	68 ± 8 c	101 ± 8 a	102 ± 10 a
EBT + skimmed milk	40 ± 10 b	53 ± 9 bc	64 ± 5 c	85 ± 4 a
75% tea + 25% water or milk				
IBT + water*	100 ± 7 a	42 ± 7 b	124 ± 2 c	93 ± 17 a
IBT + whole milk	41 ± 6 b	37 ± 8 b	60 ± 6 b	34 ± 4 b
60% tea + 40% water or milk				
IBT + water*	100 ± 3 a	42 ± 9 b	96 ± 9 a	86 ± 14 a
IBT + whole milk	37 ± 9 b	50 ± 8 bc	67 ± 9 c	62 ± 9 c
with stabilization				
75% tea + 25% water or milk				
IBT + water*	100 ± 6 c	79 ± 6 f	33 ± 5 a	31 ± 4 a
IBT + whole milk	38 ± 2 ad	44 ± 1 d	20 ± 1 h	24 ± 2 ah
IBT + semiskimmed milk	38 ± 3 ad	45 ± 2 d	22 ± 2 h	26 ± 1 ah
IBT + semiskimmed milk + sucrose	53 ± 4 e	60 ± 3 g	28 ± 3 ah	31 ± 3 a
60% tea + 40% water or milk				
IBT + water*	100 ± 7 c	82 ± 6 e	34 ± 3 a	33 ± 3 a
IBT + whole milk	36 ± 3 a	46 ± 3 f	23 ± 1 dgh	26 ± 1 adh
IBT + semiskimmed milk	38 ± 3 a	44 ± 1 f	24 ± 1 dgh	29 ± 2 adh
IBT + semiskimmed milk + sucrose	37 ± 3 a	46 ± 2 f	24 ± 1 dgh	27 ± 0 adh

[a] Each tea formulation is compared to the tea with water control with equal tea concentration; controls marked with an asterisk (*) are set to 100%. Tea, before digestion; G, after gastric phase; G&I, after both gastric and intestinal phases; G&I&BBM, after all three digestive phases. Subsets, not sharing a common letter, are significantly different as analyzed by one-way ANOVA using Newman−Keuls multiple-comparison post-test ($p < 0.001$, $p < 0.01$, or $p < 0.05$) . For details about the stabilization protocol see the text.

BBM digestion of stabilized and not stabilized IBT are comparable ($p > 0.05$).

Effect of in Vitro Digestion of Tea without Milk on Catechin Recovery. Nonstabilized samples of EBT and IBT + water show similar TCAT levels during all digestive stages except after the gastric phase, when a decrease in TCAT is observed (Table 2). Stabilized samples of IBT + water show a decrease in TCAT during the whole digestion period; however, the level of TCAT is consolidated after gastrointestinal digestion at about 33%. This is comparable to TCAT in GT after in vitro digestion, when about 36% remained.[28]

Effect of in Vitro Digestion of Tea with Milk and Sugar on Catechin Recovery. EBT with either 5.6 or 15% of skimmed milk (without stabilization, Table 2) showed some liberation of TCAT during all digestive steps, resulting in results comparable to those of the EBT control after in vitro digestion ($p > 0.05$).

IBT with either 25 or 40% milk (Figure 2, panels A and B, respectively) showed some liberation of TCAT during all digestive steps. TCAT concentrations in IBT with milk were comparable to the IBT + water control after gastrointestinal and BBM digestion ($p > 0.05$). The type of milk, either semiskimmed milk or whole milk, used in IBT formulations did not influence the obtained results of TCAT during all digestive stages ($p > 0.05$).

Effect of Milk Amount and Type and Sugar on Catechin Recovery of Digests. Our simulated digestion results of EBT and IBT with 5.6−40% milk show that the TCAT level after in vitro digestion of BT with milk is similar to the TCAT level of plain BT after in vitro digestion. We found that TCAT concentrations in IBT with milk, irrespective of fat content, were comparable to those in the IBT + water control after gastrointestinal and BBM digestions (Figure 2, $p > 0.05$). To date, no data are available about catechin levels after digestion of IBT with 25 and 40% milk. Green et al.[28] described the addition of whole milk (10−50%) to GT, which showed a positive effect on the in vitro digestive recovery of TCAT.

Addition of sucrose (40 g/L) shows a stabilizing effect, which is still present after gastric simulation of IBT with 25% semiskimmed (Figure 2A, $p < 0.001$). However, after gastrointestinal digestion and BBM digestion, in vitro digestive recovery of TCAT is not significantly enhanced by sucrose (Figure 2A, $p > 0.05$) using the current analysis conditions. It is unclear at this stage if sucrose is also stabilizing TCAT during digestion when lower amounts of milk are added to tea.

Recently, Green et al.[28] described that GT combined with citrus juice enhances the in vitro digestive recovery of TCAT and increases uptake efficiency in Caco-2 cells.[39] This phenomenon could not be explained by the ascorbic acid level, indicating that other factors from juice may be responsible. From the same group Peters et al.[40] described that a formulation of GT with sucrose (without milk) modestly but significantly improved overall catechin digestive recovery, which supports our findings.

Besides catechins, other in vivo levels of flavonoids, such as the flavonols quercetin and kaempferol, are not affected by the addition of milk to tea.[41] In comparison, addition of whole milk to coffee did not alter the overall bioavailability of coffee polyphenols, determined via the phenolic acids in human plasma.[42]

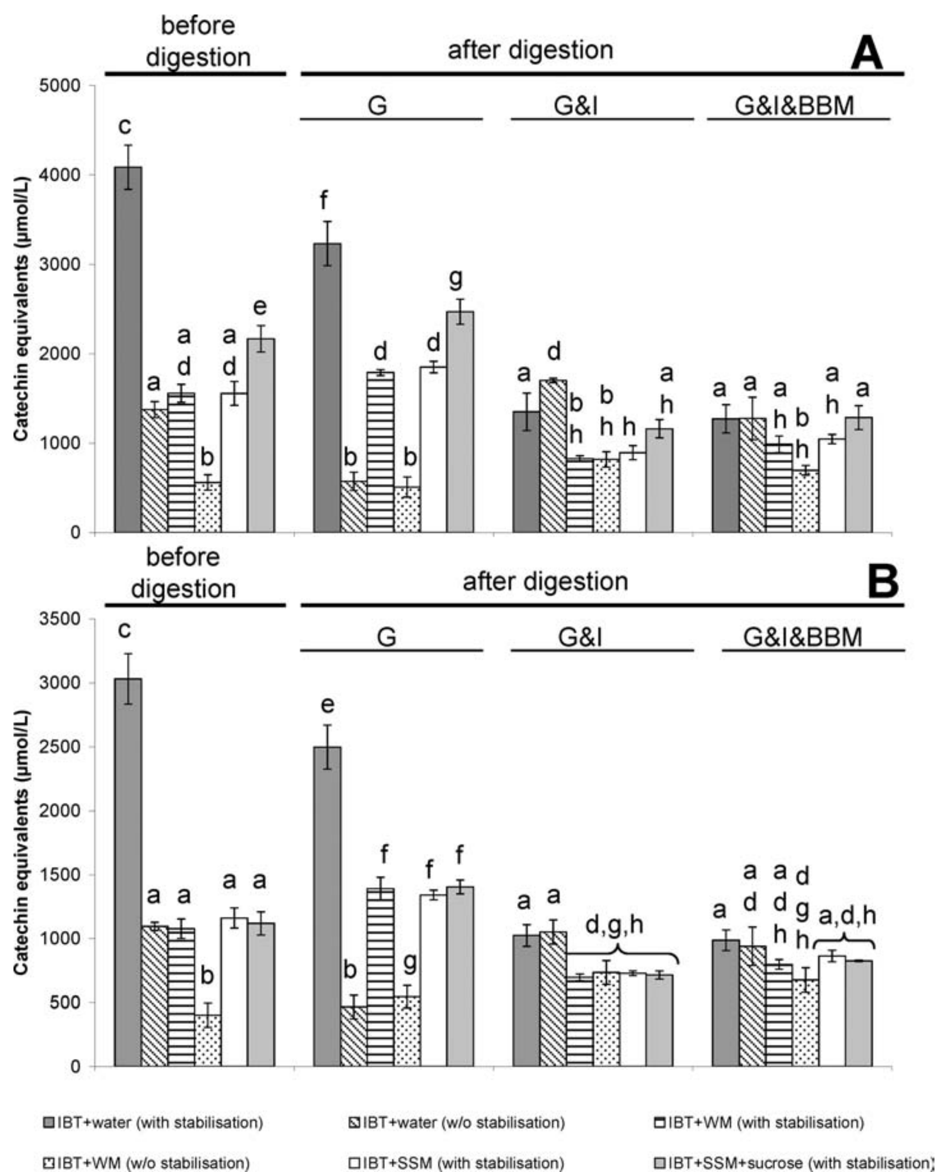

Figure 2. Recovery of TCAT in the soluble fraction of (A) 75% IBT + 25% water or milk and (B) 60% IBT + 40% water or milk. Brewed tea is before digestion, G is after gastric phase, G&I is after both gastric and intestinal phases, G&I&BBM is after all three digestive phases (±SEM, $n = 3$). WM is whole milk, SSM is semiskimmed milk, and SM is skimmed milk. Bars not sharing a common letter are significantly different as analyzed by one-way ANOVA using Newman−Keuls multiple-comparison post test ($p < 0.001$, $p < 0.01$, or $p < 0.05$).

Whole milk added to cocoa resulted in similar plasma levels of cocoa polyphenols[43] and showed no influence on the concentrations of polyphenol metabolites in plasma and urinary secretion in the studies of Roura et al.[44,45] Also, Mullen et al. showed that the addition of milk to commercial cocoa did not influence concentrations of polyphenol metabolites in plasma; however, urinary excretion of polyphenol metabolites was lowered.[44,46]

Impact of Protein Digestion. Horizontal SDS electrophoresis of IBT was performed to visualize the cleavage of tea catechin−milk protein complexes by proteolytic enzymes during simulated digestion (Figure 3). Protein patterns of plain whole cow's milk, 25 and 40%, before digestion (Figure 3, lanes 1 and 4, respectively) show the caseins and both whey proteins, α-lactoalbumin and β-lactoglobulin. The applied dilution of plain IBT (75 and 60%) with water (25 and 40%) is too low to visualize the proteins naturally present in tea (Figure 3, lanes 2 and 5, respectively).

IBT (75 and 60%) with whole cow's milk (25 and 40%) before digestion (Figure 3, lanes 3 and 6, respectively) shows protein patterns similar to those of the milk controls but with lower intensity, suggesting that not all amino acids of the milk protein in IBT with milk are available for staining. The difference in intensity between IBT with 25% milk and IBT with 40% milk is due to the higher amount of protein available in 60% IBT with 40% milk.

After gastric simulation, the breakdown pattern of caseins is present in both milk controls and IBT with milk. IBT with 25% milk shows much less intensity of staining than 25% milk control (Figure 3, lane 10 vs 8), due to interactions of protein with tea, but all caseins are broken down to smaller proteins. More proteins are available for staining in IBT with 40% milk, but the intensity is still lower than that of the milk control (Figure 3, lane 13 vs 11).

After intestinal simulation, only a tiny amount of small proteins is present in both milk controls and IBT with milk,

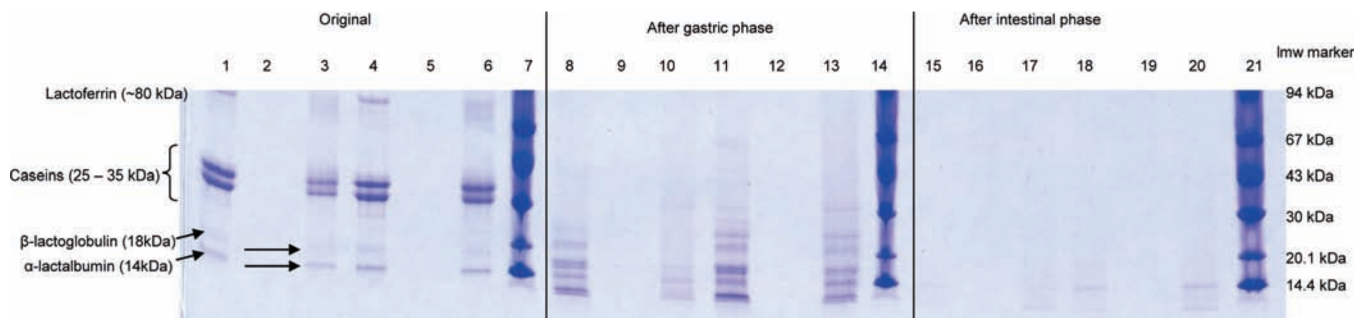

Figure 3. SDS electrophoresis of Indian tea samples with and without milk. Low molecular weight (lmw) markers (GE Healthcare) are present in lanes 7, 14, and 21 and specified in lane 21. Protein patterns of samples before digestion are shown in lanes 1−6, after gastric simulation in lanes 8−13, and after the intestinal simulation in lanes 15−20. Samples were as follows: lanes 1, 8, and 15 = 25% v/v plain bovine milk in water; lanes 4, 11, and 18 = 40% v/v plain bovine milk in water. Lanes 2, 9, and 16 show 75% IBT + 25% water, and lanes 5, 12, and 19 show 60% IBT + 40% water. Finally, lanes 3, 10, and 17 show 75% IBT + 25% milk, and lanes 6, 13, and 20 show 60% IBT + 40% milk.

because all large proteins are broken down to small proteins and peptides. Slightly more proteins are still present in the IBT with milk samples compared to the controls (Figure 3, lane 17 vs 15 and lane 20 vs 18). It could be that the proteolytic enzymes present in the simulated digestion have slightly more difficulty cleaving proteins of milk in the presence of IBT due to catechin−milk protein interactions.

No samples after BBM digestion were analyzed using gel electrophoresis because only peptides and amino acids smaller than 10 kDa are present after BBM digestion, which cannot be visualized using the described gel electrophoresis conditions.

In conclusion, this study shows that the in vitro bioaccessible TCAT fraction of tea (either EBT or IBT) with milk is influenced neither by the brewing method or fat content nor by the amount of milk. Furthermore, we showed that tea catechin−milk protein complexes are easily hydrolyzed by proteolytic enzymes during the simulated digestion.

To date, only two groups describe their in vitro digestion experiments with tea and milk. Both groups found equal[27] or even enhanced levels of catechin[28] after digestion of GT with milk compared to digestion of GT without milk. This is the first described study with in vitro digestion results of BT with milk, and we find that the digestive catechin recoveries of both EBT and IBT with milk (5.6−40%) are similar ($p > 0.05$) to the digestive catechin recovery of EBT and IBT without milk. Conclusions from in vitro interaction studies performed with tea and milk without digestion should therefore be read with care, especially if complete or soluble fractions of tea polyphenol−milk protein complexes are added to in vitro endothelial cell assays.

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Notes

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ABBREVIATIONS USED

AOX, antioxidant; BBM, brush border membrane; BT, black tea; DMACA, *p*-dimethylaminocinnamaldehyde; EBT, English black tea; GT, green tea; IBT, Indian black tea; LMW, low molecular weight; TCAT, total catechin.

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