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## Cow's milk as a dietary source of equol and phenolic antioxidants: differential distribution in the milk aqueous and lipid fractions

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**Abstract** Cow's milk contains bioactive secondary phenolic compounds that are formed by bovine's gut bacterial flora from plant phenolic compounds. Equol, a metabolite of daidzein, is one such secondary compounds of biological interest. The potential of phenolic compounds in milk as dietary antioxidants and their distribution within the milk matrix were examined. The equol concentrations, total phenolic contents, and antioxidant activity (Trolox equivalent antioxidant capacity and inhibition of F<sub>2</sub>-isoprostanes formation by human neutrophils *in vitro*) were measured and compared in commercial cow's milk of varying lipid contents. The Trolox equivalent antioxidant capacity and ability to inhibit F<sub>2</sub>-isoprostanes formation *in vitro* increased with the concentrations of phenolic compounds and equol in the milk. Equol and total phenolic compound concentrations correlated positively with the milk lipid concentrations. Separate experiments using homogenized and non-homogenized milk showed that dairy equol and phenolic compounds distributed to larger extent in the lipid fraction than in the aqueous fraction of cow's milk. Our results showed that cow's milk may serve as a dietary source of unique phenolic compounds, such as equol. Skimming of cow's milk may reduce the original amount of equol and phenolic compounds and thereby diminishes the overall nutritive value and functional properties of cow's milk.

**Keywords** Cow's milk · Equol · Phenolic compounds · Antioxidant capacity · F<sub>2</sub>-isoprostanes

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## 1 Introduction

Phenolic compounds (PCs) are natural dietary phytochemicals that are widespread in the human diet (Manach et al. 2005). Dairy cow's milk is shown in recent studies to contain appreciable amounts of equol, a polyphenolic metabolite of daidzein (Hoikkala et al. 2007; Mustonen et al. 2009). These PCs are obtained as part of the cow's diet from plant feed (Mustonen et al. 2009). In addition, PCs of bovine origin are formed by the action of gut bacterial flora on plant PCs, and secondary metabolites, such as equol, can be found in dairy cow's milk (Mustonen et al. 2009). The physicochemical properties of equol have been summarized in Table 1. Equol has demonstrated antioxidant property by inhibiting reactive oxygen species (ROS) and increasing nitric oxide productions in vitro (Chung et al. 2008; Hwang et al. 2003).

Milk is an emulsion of lipid globules dispersed in a continuous aqueous phase (Singh and Sachan 2011). The partition of molecules and ions between the milk lipid and aqueous phases can be explained, in great part, by their hydrophilic or lipophilic characters, which in turn are determined by their chemical structures. PCs possess both hydrophilic and lipophilic characteristics in their chemical structures (MacDonald and Griffiths 2010). Depending on molecular size and geometry, milk PCs may form stronger intermolecular attraction or repulsion either with lipids or aqueous components. Presently, little is known about the biological relevance and distribution of the milk PCs in fresh cow's milk.

Our study measured and compared the equol and total PC concentrations in fresh commercial dairy cow's milk of varying lipid contents. We hypothesize that equol and PCs are present in sufficiently high concentrations in commercial dairy cow's milk in order to exert antioxidant capacity. We also hypothesize that equol and PCs are preferentially associated with the milk lipids than with the aqueous matrix.

## 2 Materials and methods

### 2.1 Chemicals and materials

Folin-Ciocalteu's reagent, methanol, sodium carbonate, gallic acid, 2,2'-amino-bis-(3-ethylbenzthiazoline-6-sulfonate), 2,2'-azobis-(2-amidinopropane)hydrochloride, concentrated sulfuric acid, copper (II) sulfate, boric acid, ammonia, sodium hydroxide, hydrochloric acid, methyl red, bromocresol green and Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), and phorbol 12-myristat 13-acetate (PMA)

**Table 1** Equol and physicochemical properties (Scifinder 2014)

Chemical name	(3S)-3-(4-Hydroxyphenyl)-7-chromanol
Chemical formula	C <sub>15</sub> H <sub>14</sub> O <sub>3</sub>
Molecular weight	242.27
Solubility (pH 7.0, 25 °C)	0.16 g.L <sup>-1</sup>
log P (octane/water)	2.77±0.32
log D (octane/water, pH 7.0, 25 °C)	2.77

were purchased from Sigma-Aldrich (MO, USA). Arachidonic acid (AA; dissolved in ethanol) and equol were purchased from Cayman Chemical (MI, USA). Fresh homogenized cow's milk of different milk lipid content classification (full cream,  $n=5$ ; low-fat,  $n=4$ ; and non-fat,  $n=3$ ) was purchased from local supermarkets in Singapore. The unpasteurized and non-homogenized cow's milk was purchased from Viknesh Dairy Farm (Singapore) with the approval from the Agri-Food and Veterinary Authority of Singapore. The non-homogenized milk was centrifuged at  $1,000\times g$  for 10 min to obtain the milk fat and non-fat (aqueous) fractions for subsequent analysis. Acetonitrile, ethyl ether, petroleum ether, and ethanol were purchased from Tedia (OH, USA).

The protein concentration in each milk was adjusted to  $30\text{ g}\cdot\text{L}^{-1}$  with distilled water before protein removal. The proteins in the milk were removed by precipitation with acetonitrile in order to eliminate their interference with analyses. Briefly, acetonitrile (3 mL) was mixed thoroughly into the milk (10 mL) before the mixture was centrifuged at  $1,000\times g$  for 10 min at  $5\text{ }^{\circ}\text{C}$ . The resulting supernatant was stored at  $-80\text{ }^{\circ}\text{C}$  for total phenolic content analysis, equol measurement, and *in vitro* experiments.

## 2.2 Total phenolic content and equol analyses

The total phenolic contents were determined using a modified Folin-Ciocalteu's method (Actis-Goretta et al. 2006).

Equol concentration in the milk was measured by liquid chromatography-ion trap-time-of-flight mass spectroscopy (LC-IT-TOF-MS) modified from a published method (Setchell et al. 2009). Briefly, the supernatant was filtered through the  $0.2\text{-}\mu\text{m}$  polyethersulfone (PES) filter before analysis. The mass spectroscopy was performed in the negative ion mode. The equol mass ion was detected at  $m/z$  241. The concentration of equol in the milk sample was determined using a standard curve obtained with known concentrations of equol.

## 2.3 *In vitro* antioxidant experiment

The antioxidant ability of milk phenolics to inhibit neutrophil-mediated AA peroxidation was measured by the formation of  $\text{F}_2$ -isoprostanes (stable marker of AA oxidation) quantitated by stable isotope labelled GC-MS (Mori et al. 1999). Briefly, human blood neutrophils were isolated from the neutrophil/erythrocyte pellet after Ficoll-Paque gradient centrifugation and dextran sedimentation of red blood cells as previously described (Loke et al. 2012). Cell viability was assessed using trypan blue exclusion and was typically  $>98\%$ . The freshly isolated neutrophils were incubated in the milk supernatant after protein extraction ( $5\times 10^6$  cells/mL milk supernatant) and AA (final concentration,  $10\text{ }\mu\text{M}$ ) at  $37\text{ }^{\circ}\text{C}$  for 5 min prior to stimulation. The cells were stimulated with PMA (final concentration,  $200\text{ nM}$ ) at  $37\text{ }^{\circ}\text{C}$  for 15 min. PMA-activated, untreated cells in 15% acetonitrile were used as positive controls, while untreated cells in 15% acetonitrile without PMA activation served as negative controls. The supernatant from the cell suspension was collected and stored at  $-80\text{ }^{\circ}\text{C}$  before  $\text{F}_2$ -isoprostanes extraction and analysis. The amount of inhibition of  $\text{F}_2$ -isoprostanes formation was expressed as a percentage of inhibition relative to the positive controls.

## 2.4 Total antioxidant capacity

The total antioxidant capacity of the milk sample was measured using a modified Trolox equivalent antioxidant capacity (TEAC) method (van den Berg et al. 1999).

## 2.5 Milk fat and protein measurement

The concentrations of fat and protein in milk sample were determined using the modified Mojonnier method and Kjeldahl method, respectively.

## 2.6 Statistical analyses

Statistical analysis of results was performed using SPSS version 15.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean±standard deviation. Between-group differences were analyzed using ANOVA followed by Bonferroni adjustment. Pearson analysis was used to determine correlation between tested variables. Results from the non-homogenized milk experiments were compared using paired *t* test. The results analyzed were considered significantly different if  $P < 0.05$  based on 95% confidence.

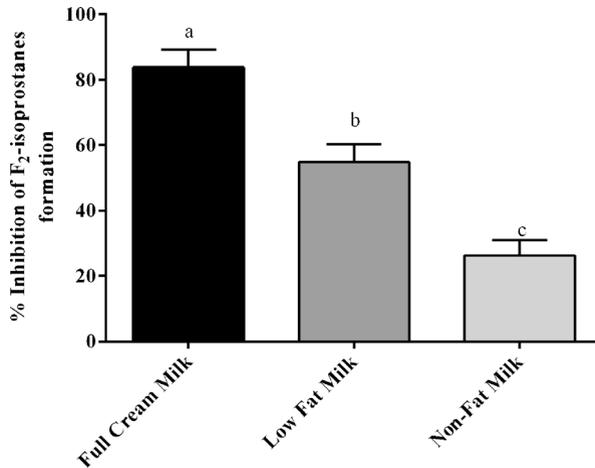
## 3 Results and discussion

Full cream cow's milks contained significantly higher amounts of equol and total phenolic compounds than their low-fat and non-fat counterparts (Table 2). The equol concentrations were significantly higher in fresh low-fat milk than in fresh non-fat milk (Table 2). The TEAC of full cream milk was significantly higher than those of low-fat and non-fat milks (Table 2). Using the measurement of F<sub>2</sub>-isoprostanes formation, an

**Table 2** Fat, protein, equol, total phenolic compounds, and Trolox equivalent antioxidant capacity in commercially available fresh dairy cow's milk and non-homogenized cow's milk

	Type of milk			
	Fresh full cream <i>n</i> =5	Fresh low-fat <i>n</i> =4	Fresh non-fat <i>n</i> =3	Non-homogenized whole milk
Fat concentration (g.L <sup>-1</sup> )	37.2±2.5 <sup>a</sup>	18.0±2.1 <sup>b</sup>	0.8±0.9 <sup>c</sup>	35.1±0.8 <sup>a</sup>
Protein concentration (g.L <sup>-1</sup> )	34.8±3.0	34.8±1.0	35.0±1.0	33.0±5.0
Equol concentration (ng.mL <sup>-1</sup> )	47.02±3.48 <sup>a</sup>	18.85±2.50 <sup>b</sup>	1.25±0.84 <sup>c</sup>	0.58±0.02 <sup>c</sup>
Total phenolic compound concentration (µg gallic acid. g <sup>-1</sup> )	0.70±0.04 <sup>a</sup>	0.26±0.18 <sup>b</sup>	0.04±0.04 <sup>b</sup>	0.70±0.06 <sup>a</sup>
Trolox equivalent antioxidant capacity (µmol Trolox L <sup>-1</sup> )	6.50±0.14 <sup>a</sup>	4.96±0.15 <sup>b</sup>	3.46±0.08 <sup>c</sup>	5.15±0.23 <sup>b</sup>

Same superscripts (a, b, c) indicate no significant difference ( $p < 0.05$ ) between different milk using ANOVA with Bonferroni post hoc adjustment



**Fig. 1** F<sub>2</sub>-isoprostane inhibition in peripheral neutrophils by full cream ( $n=5$ ), low-fat ( $n=4$ ) and non-fat ( $n=3$ ) milk extracts. Bars with different letters (*a*, *b*, *c*) are significantly different to one another (ANOVA)

established stable marker of oxidative stress, our results showed that commercial milk exerted significant antioxidant activity *in vitro*, with full cream milk exerting significantly stronger activity than low-fat and non-fat milk (Fig. 1). The observed significant positive correlations (TEAC—*inhibition of F<sub>2</sub>-isoprostanes formation*:  $r=0.814$ ,  $P<0.0001$ ; total PCs—TEAC:  $r=0.9941$ ,  $P<0.0001$ ; total PCs—*inhibition of F<sub>2</sub>-isoprostanes formation*:  $r=0.810$ ,  $P<0.0001$ ; equol—TEAC:  $r=0.7422$ ,  $P<0.0001$ ; equol—*inhibition of F<sub>2</sub>-isoprostanes formation*:  $r=0.950$ ,  $P<0.0001$ ) suggested that the observed antioxidant activity may be attributed to the phenolic compounds naturally present in the milk. The majority of PCs indigenous in cow's milk is thought to derive from the feed, although a proportion may be the products of amino acid catabolism (Lopez and Lindsay 1993). Transformations, whether in the guts or metabolically, produce unique secondary dairy PCs, which are different from the indigenous ones present in the feeds. These secondary dairy PCs are usually absent in the traditional sources of PCs in the human diet, such as fruits and vegetables, but may exert significant biological activity *in vivo*, for example, antioxidant and anti-inflammatory activities. Equol is one such PC, which is not naturally present in human diet but is produced naturally in the gut of certain individuals by the bacterial biotransformation of daidzein, an isoflavone (Setchell and Clerici 2010). Most people produce equol after consumption of daidzein-containing foods or supplements, but there is only about 30 to 40% of the population who are able to convert daidzein to produce sufficient equol to achieve a urinary log<sub>10</sub> equol/diadzein ratio  $>1.75$  and be classified as equol producers (Setchell and Cole 2006). Equol production *in vivo* has been associated with a positive outcome for low-density lipoprotein cholesterol, endothelial function, and C-reactive protein in intervention studies (Clerici et al. 2007; Setchell and Clerici 2010). Equol treatment protected against H<sub>2</sub>O<sub>2</sub>-induced cell death by the reduction of intracellular ROS production (Chung et al. 2008). Equol inhibited cell-mediated low-density lipoprotein modification *in vitro* by inhibiting superoxide production and increasing nitric oxide availability (Hwang et al. 2003). Equol is also a selective estrogen receptor modulator with its associated health benefits and risks (Axelson et al. 1982; Setchell

and Clerici 2010). The risks associated with elevated in vivo equol concentration must be considered. Future human intervention studies should examine possible biological benefits and risks of equol. While previous studies had examined the levels of equol in organic cow's milk (Hoikkala et al. 2007) or milk from cows fed with clover (Mustonen et al. 2009), our results reported the levels of equol in cow's milk from the store shelf, though our reported levels were comparatively lower. As there is presently no reported dietary source of equol, the presence of significant amount of equol in cow's milk may present the latter as a dietary source of equol, thereby expanding the therapeutic benefits of equol to the equol non-producers. Our findings are supported by a recent study reporting significant correlation between dairy consumption and urinary equol concentration in a representative sample of more than 3,000 adults (Frankenfeld 2011). Further studies are required to identify unique phenolic compounds in cow's milk as well as to elucidate potential biological activity of these compounds in relation to the human health and well-being.

The protein concentrations were similar in all tested milk (Table 2). As expected, fat content was significantly higher in fresh full cream milk compared to low-fat milk, which in turn had significantly higher fat content than fresh non-fat milk (Table 2). Equol and PC concentrations were found to be correlated significantly with the milk fat content (equol:  $r=0.9900$ ,  $P<0.0001$ ; PC:  $r=0.7903$ ,  $P<0.001$ ). The associations were absent with milk protein concentrations (data not shown). Significant positive correlation was also observed between the equol and PC concentrations in these milks ( $r=0.729$ ,  $P<0.0001$ ). The non-homogenized milk contained comparable amounts of fat and protein as the homogenized milk samples used in our experiments (Table 2). The fat concentration was higher in the fat fraction than in the aqueous fraction of the non-homogenized milk ( $140.2\pm 3.1$  vs  $7.0\pm 0.2$  g.L<sup>-1</sup>,  $P<0.05$ ). The two fractions differ significantly in protein concentrations ( $8.3\pm 1.2$  in fat fraction vs  $123.8\pm 18.6$  g.L<sup>-1</sup> in aqueous fraction,  $P<0.05$ ). The milk fat fraction in non-homogenized milk contained significantly higher amounts of PCs ( $2.42\pm 0.20$  vs  $0.20\pm 0.02$  µg gallic acid/g,  $P<0.05$ ) and equol ( $2.27\pm 0.09$  vs  $0.13\pm 0.01$  ng.mL<sup>-1</sup>,  $P<0.05$ ) than the corresponding aqueous fraction. Significantly higher TEAC was observed in the fat fraction than the corresponding aqueous fractions prepared from the non-homogenized milk ( $3.81\pm 0.24$  vs  $1.46\pm 0.18$  µmol Trolox L<sup>-1</sup>,  $P<0.05$ ). The fat and aqueous fractions retained 83.7±5.2 and 26.9±5.2% of the F<sub>2</sub>-isoprostane inhibition ability of the whole non-homogenized milk. These experiments with homogenized and non-homogenized milks showed that equol and milk PCs are unevenly distributed between the milk fat and aqueous fractions. Whereas the mode of interactions between milk PCs and lipid molecules remains largely unknown, equol and PCs seem to form stronger interactions with the non-polar lipid molecules than the polar constituents in the aqueous fraction. Our correlation results suggest that equol and milk PCs may be removed together with milk fat during the skimming process, thereby reducing the functional value of the original product.

Our results are limited to milk available to the consumers from the local store. We have no information about the sample history and feeding practice of the cow, which were shown to influence the equol and phenolic contents in the milk.

## 4 Conclusions

Our studies reported that cow's milk available in stores contains considerable amounts of equol and PCs, and the latter exerts significant antioxidant activity *in vitro*. Considerable amount of these PCs may be removed along with the milk fat during skimming. Cow's milk may provide a distinct profile of bioactive secondary PCs, such as equol, for the human diet which are not naturally present in most fruits and vegetables.

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**Conflict of interest** The authors declare that they have no conflict of interest.

**Compliance with ethics guideline** This article does not contain any studies with human or animal subjects performed by any of the authors.

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