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The effect of commonly used dairy processing techniques and unit operations on the equol content of dairy products

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ABSTRACT

Since a large portion of the milk consumed by human is processed, the aim of this study was to determine possible changes in equol content upon processing milk. Individual milks with different levels of equol content were collected from the morning milking to make skimmed milk and cream, as well as pasteurised and sterilised full fat milks. Pasteurised milk was further processed into yoghurt, kefir, cottage cheese and whey. Yoghurt and kefir were also produced from sterilised milk. Equol content was not affected by either pasteurisation or sterilisation. Compared with raw milk, a higher concentration of equol was measured in skimmed milk, but equol content in cream was lower. The concentration of equol remained unchanged after yoghurt production but was reduced by more than 50% after kefir production. The equol content in whey was low compared with that of raw milk, while it increased sharply in cottage cheese.

1. Introduction

Phytoestrogens are a large group of polyphenolic plant secondary metabolites, with structural similarities to mammalian oestrogen. These molecules can bind to oestrogen receptors and induce an oestrogenic or antioestrogenic effect (Atkinson, Frankenfeld, & Lampe, 2005; Kuhnle et al., 2008; Setchell, Brown, & Lydeking-Olsen, 2002; Steinshamn, Purup, Thuenand, & Hansen-Møller, 2008). Isoflavones are one of the major classes of phytoestrogens and can be found in both grain legumes such as soya, and grassland legumes including red clover.

Upon ingestion by cows, these molecules can be either transferred to the milk or transformed into metabolites by microorganisms from rumen and intestine. Equol, one of the dominating metabolites in milk (Njåstad et al., 2014), results from bacterial metabolism of two isoflavones: daidzein and formononetin. Formononetin is first metabolised to daidzein and subsequently to equol (Setchell et al., 2002). Antignac, Cariou, Le Bizec, and André (2004) and Daems, Jasselette, Romnée, Lognay, and Froidmont (2015) found equol in various types of commercial milks. This suggests that equol is naturally present in milk of cows feeding practical diets because they often contain legumes forage or leguminous plants.

Equol is more bioactive than its precursors and could have diverse pharmacological properties like anticarcinogenic, antimutagenic and antioxidant activities, which contribute to a reduction in hormone-dependent cancers, heart diseases, osteoporosis or menopausal symptoms (Atkinson et al., 2005; Jackson, Greiwe, & Schwen, 2011; Setchell et al., 2002; Steinshamn, Purup, Thuen, & Hansen-Møller, 2008). A dose of 10 mg d^{-1} of equol is often used in medical studies, but it remains unclear whether this is necessary or sufficient to affect these diseases or symptoms (Takeda, Shiina, & Chiba, 2018). A dose sufficient to prevent these is also not defined. Yee et al. (2008) reported a non-observable adverse effect level (NOAEL) of 2000 mg kg^{-1} d^{-1} of equol in rats but no study was done in human.

In contrast to cattle, only a small proportion of humans in Western countries (30–50%) can produce equol (Atkinson et al., 2005; Mustonen et al., 2009). For the non-producers, Setchell et al. (2002) suggested that an oral administration of equol could be an alternative strategy to obtain the benefit of this molecule. Bovine milk and dairy products contain equol and are therefore potential dietary sources of natural equol for non-producers (Kuhnle et al., 2008; Mustonen et al., 2009; Steinshamn et al., 2008).

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Different studies showed that red clover or soybean meal in dairy cattle feed induced equol in raw milk (Höjer et al., 2012; Kašparovská et al., 2017; Křížová et al., 2011b; Křížová, Třináctý, Hajšlová, & Havlíková, 2011a; Mustonen et al., 2009; Steinshamn et al., 2008). However, in developed countries, consumer preferences tend towards processes dairy products (OECD, 2017). The aim of this study was to determine the effect of commonly used dairy processing techniques and unit operations on equol content in major dairy products. Animals in this study were fed with two diets containing different sources of isoflavones: soybean meal or red clover silage, which are often used on dairy farms and led to milk with different equol content levels.

2. Materials and methods

The experiment was conducted at the Walloon Agricultural Research Centre, 5030 Gembloux, Belgium and followed the recommendations on care and use of laboratory animals developed by the University of Liege, Belgium (The experimental protocol approved by the ethical committee was 15–1766).

2.1. Animals and diets

The experiment was carried out using four Holstein cows (2.3 ± 1.8 number of lactation; 123 ± 48 days of lactation) with similar milk production (29.7 ± 2.2 kg d^{-1}), divided into two groups according to calving date and parity. Two iso-dry matter (iso-DM), iso-nitrogen and iso-net energy diets (Table 1), with the same forage to concentrate ratio (80:20) were formulated to enable a daily milk production of 29 kg d^{-1} according to Dutch Feeding Standards (Tamminga et al., 1994; Van Es & Van der Honing, 1977). Diets were dispensed at an intake level close to ad libitum. One diet (SBM) contained only soybean meal as a source of isoflavones while in a second diet (RC) these molecules were supplied exclusively by red clover silage (*Trifolium pratense* L., 2nd cut in May 2015, 57 days of regrowth, 3 days of wilting). The experiment was carried out in the form of a cross-over design and was divided into two 21-day periods. The periods consisted of a 15-d preliminary period of adaptation to the diets and a 6-d sampling period. Cows were fed individually twice a day (7.00 and 17.00 h) and had a free access to water.

Table 1
Composition of soybean meal diet (SBM) and red clover diet (RC) fed to the dairy cows.[a]

Parameter	SBM	RC
Component (kg DM d^{-1} cow^{-1})		
Maize silage	9	9
Ray grass silage	7	0
Red clover silage	0	7
Soybean meal	2.70	0
Dried sugar beet pulp	1.30	2.05
Corn gluten meal	0	0.95
Rapeseed meal	0	1
Blend minerals and vitamins	0.25	0.25
Sodium bicarbonate	0	0.15
NaCl	0.07	0
Total (kg DM d^{-1} cow^{-1})	20.3	20.4
Crude protein (g kg^{-1} DM)	143	144
Net energy (kcal kg^{-1} DM)	1465.2	1480.1
Digestible protein (kg DM d^{-1} cow^{-1})	79	79
OEB (kg DM d^{-1} cow^{-1})	0	8

[a] Blend minerals and vitamins was Bovimin 21/7 (Dumoulin, Seilles, Belgium), composition: Ca, 21%; P, 7%; Na, 1%; Mg, 5%; Cu, 1000 mg kg^{-1}; Mn, 1350 mg kg^{-1}; Zn, 2500 mg kg^{-1}; I, 150 mg kg^{-1}; Vit E, 2000 mg kg^{-1}; Vit A, 900,000 UI kg^{-1}; Vit D3, 200,000 UI kg^{-1}. Net energy and digestible protein were calculated according to the Dutch system (Tamminga et al., 1994; Van Es & Van der Honing, 1977). OEB is the difference between the amount of microbial raw protein based on rumen available energy and the amount of microbial raw protein based on rumen available nitrogen (Tamminga et al., 1994).

During the sampling periods, refusals were collected and weighed daily after drying (60 °C, 48 h) and analysed to estimate intake. Feed samples were analysed to determine the concentration of daidzein and formononetin. They were freeze-dried for 48 h (Martin Christ Delta 1–24 LSC plus, Analis, Suarlée, Belgium), then ground in a Cyclotech mill (1 mm screen) (FOSS Electric, Hillerød, Denmark) and stored frozen (−20 °C) under vacuum until analysis.

2.2. Sampling and manufacturing of dairy products

Cows were milked twice a day (6.30 and 16.30 h), just before feeding. Milk yield was recorded on 5 consecutive days at each milking. Five milk samples were collected on a daily basis for each cow. To do so, representative samples from evening and following morning milking were mixed in proportion corresponding to milk yield. One sub-sample was directly analysed by infrared milk analyser (Foss Milkoscan FT6000, Foss Electric) to determine fat and protein content in milk. Another sub-sample was frozen to −20 °C immediately after the collection to allow for a subsequent equol content analysis.

On the sixth day, individual milks from the morning milking were fully collected for technological processing. A 25 mL sample was immediately frozen for equol analysis before milk transformation (raw milk). After homogenisation, 1 L was sampled and centrifuged on EleCrem 1 (Elecrem, Fresnes, France) to separate cream from skim milk, 3 L were pasteurised at 65 °C for 30 min and 3 L were sterilised at 120 °C for 20 min. Yoghurt and kefir were prepared with pasteurised (PA) and sterilised (ST) milk. Cottage cheese was prepared with PA milk. Yoghurt was made by adding 15 g of ferment B8 (Walloon Agricultural Research Centre) to 0.5 L of milk. The mixture was then incubated at 44 °C for 2.30 h before cooling to 4 °C. Kefir was made by adding 15 g of grain KJ (Walloon Agricultural Research Centre) composed as described by Ninane (2008) to 0.25 L of milk. The mixture was then incubated at room temperature for 24 h, before filtering and stored at 4 °C for 24 h. Cottage cheese was made by adding 15 g of Flora Danica ferment (originally Chr. Hansen, Copenhagen, Denmark; replicated since 1993) to 1 L of milk. The mixture was incubated for 24 h at room temperature and drained with butter muslin for 12 h at 4 °C and whey was sampled. Cottage cheese was weighed to calculate cheese yield. Samples of each product (yoghurt PA, yoghurt ST, kefir PA, kefir ST, cottage cheese PA and whey PA) were stored frozen at −20 °C until equol content was analysed.

2.3. Analytical procedures

2.3.1. Isoflavone quantification in feed

The isoflavone concentration was determined following the method optimised and validated by Daems et al. (2016). Each feedstuff sample was thawed at ambient temperature and analysed in duplicate. About 500 mg of each sample were mixed with 25 mL of H_2O:methanol (45:55, v/v) (methanol (LC-MS) from J.T. Baker, Devender, The Netherlands) and extracted over 10 min using an ultrasonic bath set at 80 °C. The mixture was then centrifuged (3200× *g*, 5 min) and 1 mL of the supernatant was evaporated to dryness. The dry residue was solubilised in 1 mL sodium acetate buffer (pH 6; Merck KGaA, Darmstadt, Germany) and 2 mL of an enzymatic mixture comprising glucosidase, glucuronidase and cellulose (Sigma Aldrich, Diegem, Belgium). Hydrolysis was conducted overnight at room temperature after which the mixture was centrifuged (3200× *g*, 5 min). The supernatant was diluted with different volumes of H_2O:methanol (40:60, v/v), according to the assumed isoflavone concentration, and 0.5 mL of diluted extract

was evaporated to dryness. Each dry residue was then reconstituted in 1 mL of H_2O:methanol (40:60, v/v) with two internal standards [50 ng mL^{-1} daidzein-d4 (C/D/N ISOTOPES, Pointe-Claire, Canada); 20 ng mL^{-1} flavone 'IS' (Sigma Aldrich)]. Reconstituted solutions were filtered (0.2 μm) and 10 μL was injected into an UPLC®-MS/MS (Waters, Zellik, Belgium) with electrospray ionisation operated in positive ionisation mode. Quantification was performed using multiple reaction monitoring. An external calibration was done with concentrations of the two targeted isoflavones ranging from 2 to 200 ng mL^{-1} H_2O:methanol (40:60, v/v). As a result of the enzymatic hydrolysis step, the isoflavone concentration determined in this study represented the sum of the conjugated and aglycone forms of the targeted compounds.

2.3.2. Equol quantification in dairy products

The equol concentration in milk and in some dairy products (yoghurt, kefir, whey) was determined following the method optimised and validated by Daems et al. (2015). Each sample was thawed overnight at ambient temperature and homogenised with a Vortex mixer (Biosan, Riga, Latvia). Aliquots of 2 mL with 0.1 mL internal standard daidzein-d4 (500 ng mL^{-1}; C/D/N ISOTOPES) and 0.1 mL β-glucuronidase (25,100 units mL^{-1}; Sigma Aldrich) were transferred into two centrifuge tubes (technical replicates). Each mixture was vortexed, placed in an oven at 37 °C and shaken continuously for 2 h. Following hydrolysis step, the mixture was cooled in an ice bath for 15 min and then centrifuged (3220× *g*, 15 min). The creamy layer and precipitate were discarded, and the liquid phase was recovered. Any fat remaining in the sample was removed by solvent extraction using n-hexane (3 × 3 mL; CARLO ERBA reagents, Val de Reuil, France). Equol was then recovered in three successive extractions using 3 mL of ethyl acetate (Biosolve, Valkenswaard, The Netherlands). Centrifugation (3220 × *g*, 4 min) was performed between each extraction. The pool of the three ethyl acetate extracts was then evaporated under vacuum at 50 °C. The residue was reconstituted in 0.5 mL H_2O:methanol (20:80, v/v) solution by vortexing for 1 min and filtering (0.22 μm). Then 10 μL were injected into the UPLC®-MS/MS (Waters) as in the isoflavone analysis performed on feeds. An external calibration was done with concentrations of equol ranging from 5 to 1000 ng mL^{-1} of H_2O:methanol (20:80, v/v).

For cottage cheese and cream, two mg of freeze-dried material were mixed with 2 mL of demineralised water before equol quantification as previously described.

2.4. Calculations and statistical analysis

Daily intakes of DM were calculated based on the difference between the amounts of DM distributed and refused. Mean daily intakes of isoflavones were calculated from the analytically determined isoflavone concentrations of individual dietary components (silage, supplemental mixture) and their respective real intakes. Mean daily production of equol was calculated from the analytically determined equol concentration in individual milks and milk daily production of each cow. Efficiency of transformation into equol was calculated by dividing daily production of equol by daily intake of isoflavone precursors.

DM intake, daily intake of isoflavones, milk yield, concentration of equol in milk and daily production of equol were subjected to ANOVA ($\alpha = 0.05$) using the mixed procedure of Minitab 17 Statistical Software (2010; Minitab Inc., State College, PA, USA. www.minitab.com). The statistical model used diet as a fixed factor and period and animals as random factors.

The effect of the different milk processing steps on equol concentration was subjected to the Student's paired samples t-test ($\alpha = 0.05$; Minitab 17). Concentrations of equol in each product (pasteurised milk, sterilised milk, skimmed milk, cream, yoghurts, kefirs, cottage cheese and whey) were compared with concentrations of equol in raw milk. The percentage of increase (or decrease) of equol concentrations was subjected to ANOVA ($\alpha = 0.05$), using the mixed procedure of Minitab 17. The statistical model used diet as a fixed factor and period and animals as random factors.

3. Results and discussion

3.1. Feed and isoflavone intake

Feed intake was slightly lower with SBM diet (Table 2) due to higher refusals of grass silage (1.12 kg d^{-1} DM ray grass silage versus 0.45 kg d^{-1} DM red clover silage, on average). Bertilsson and Murphy (2003) and Steinshamn (2010) have shown that cows fed to an intake level close to ad libitum ingest more red clover silage than ray grass silage due to rumen kinetics, which is influenced by the fibre fraction of diets.

Total intake of isoflavones differed significantly according to the diet (Table 2). Soybean meal contained 667 mg kg^{-1} DM daidzein and only traces of formononetin, while red clover silage contained more equol precursors with, 764 and 190 mg kg^{-1} DM of formononetin and daidzein, respectively. Furthermore, red clover silage could be incorporated into the diets in a larger amount than soybean meal (7.0 kg DM versus 2.7 kg DM) while complying with dietary recommendations. Consequently, as expected, cows were eating over three times the quantity of equol precursors with the RC diet than with the SBM diet.

3.2. Milk production and equol concentration in milk

Diet had no effect on milk production or on the fat and protein contents (Table 3). Consequently, it had no effect on fat protein corrected milk (FCPM), but it significantly influenced the equol content of milk, which was higher with the RC diet than with the SBM diet (282.3 μg kg^{-1} versus 46.9 μg kg^{-1}).

In addition to the influence of dietary intake of equol precursors on equol concentration, a pronounced animal effect was also recorded on isoflavone intake and on both equol content and daily production (Tables 2 and 3). Individual differences in equol content may be explained by specific rumen microflora, passage rate of digesta or by renal clearance of equol (Höjer et al., 2012).

3.3. Effect of processing

Compared with raw milk, equol content in skimmed milk was increased by 30.5% (Fig. 1) without any impact from the diets received by cows ($P = 0.91$). Accordingly, equol concentration in cream was significantly lower (−62.8%) and no difference between diets was observed ($P = 0.244$). This indicates a higher affinity of equol for the aqueous fraction. Antignac et al. (2004) and Daems et al. (2015) found no difference between skimmed milk (66.7 μg kg^{-1}; 22.4 μg kg^{-1}) and raw milk (69.7 μg kg^{-1};

Table 2
Effects of feeding cows with soybean meal diet (SBM) or red clover diet (RC) on dry matter intake and isoflavone intake.[a]

Component	Diet			*P*-value	
	SBM	RC	SEM	Diet	Animal
Dry matter intake (kg d^{-1})	19.12	19.89	0.21	<0.001	<0.001
Isoflavones intake (mg d^{-1})					
Daidzein	1883.0	1321.6	52.6	<0.001	<0.001
Formononetin	7.0	5235.4	428.0	<0.001	<0.001
Total of precursors	1890.0	6557.0	391.0	<0.001	<0.001

[a] *P*-value is for ANOVA test using diet as a fixed factor and period and animals as random factor.

Table 3
Effects of feeding cows with soybean meal diet (SBM) or red clover diet (RC) on milk yield, milk composition and equol content in milk.[a]

Parameter	Diet			*P*-value	
	SBM	RC	SEM	Diet	Animal
Milk yield (kg d^{-1})	28.1	28.1	0.47	0.905	<0.001
FPCM yield (kg d^{-1})	27.2	27.7	0.37	0.531	0.082
Fat (g kg^{-1} of milk)	39.0	40.4	1.10	0.292	<0.001
Protein (g kg^{-1} of milk)	28.9	29.3	0.53	0.557	<0.001
Equol (μg kg^{-1} of milk)	46.9	282.3	27.6	<0.001	<0.001
Daily production of equol (μg d^{-1})	1332.5	7687.7	699.0	<0.001	<0.001

[a] Abbreviation: FPCM, fat protein corrected milk, determined as follows: FPCM = [0.337 + (0.116 × %fat) + (0.06 × %protein) × milk yield]. *P*-value is for ANOVA test using diet as a fixed factor and period and animals as random factor.

34.6 μg kg^{-1}) but their samples came from commercial milks and were not paired. The difference between skimmed milk (26.5 μg kg^{-1}) and raw milk (24.6 μg kg^{-1}) observed by Křížová et al. (2011a) was smaller than ours. These authors also observed a lower concentration in cream (17.5 μg kg^{-1}) than in raw milk. Kuhnle et al. (2008) found no equol in commercial cream and similar concentrations in commercial skimmed or semi-skimmed milk.

As observed by Křížová et al. (2011a, b), pasteurisation and sterilisation had no effect on milk equol concentration ($P = 0.23$ and $P = 0.72$ respectively). This confirms that equol is not affected by heat treatment up to 120 °C. The commercialisation and consumption of raw milk are not recommended because of its microbial flora, which could contain some pathogens like *Listeria* sp. (EFSA, 2015). This being the case, humans are drinking more pasteurised or sterilised milk than raw milk. This appears to have no impact on their equol intake.

Mean equol contents of yoghurts were not significantly different from those of the initial milks, for both the PA and ST milk types ($P = 0.56$ and 0.36, respectively). Kašparovská et al. (2017) also observed no effect of processing yoghurts on equol concentration. Accordingly, Křížová et al. (2011a) found similar concentrations in yoghurts and milks (25.9 μg kg^{-1} and 25.3 μg kg^{-1}, respectively). Consequently, equol content remains unaffected by processing steps involved in yoghurt manufacturing.

Compared with raw milk, equol concentration in kefir decreased by 65.1% and 58.3% respectively when made from PA or ST milk. Diets had no impact on the decrease observed during kefir production ($P = 0.52$ and $P = 0.76$, when kefir was made with PA and ST milk, respectively). To our knowledge, no study has been published on the equol concentration in kefir, or on the ability of kefir microorganisms to metabolise equol. A decrease in polyphenol concentration during fermentation of cocoa beans has been described by Albertini et al. (2015) and Camu et al. (2008). Romero, Brenes, García, García, and Garrido (2004) observed an increase or a decrease in polyphenol concentration during the fermentation of black olives, depending on the type of molecule. We therefore hypothesise that microbial activities occurring during kefir production could affect the equol content and led to a reduction of this metabolite. More studies should be conducted to confirm this result and identify the mechanisms involved.

Finally, equol was more concentrated in cottage cheese (+150.2%) and less concentrated in whey (−68.7%) when compared with raw milk. The proportions of equol eliminated in whey ($P = 0.425$) or remaining in cheese ($P = 0.056$) were not significantly influenced by the diet. With a different process, Křížová et al. (2011b) also found lower concentration in whey but around the same concentrations in cheese and milk. During our process, with a cottage cheese yield reaching 37.6%, we observed that the percentages of equol (87.7%) and protein (81.2%) in milk remaining in cheese were similar ($P = 0.61$). These data suggest that equol could bind to proteins, and mainly to casein. Interestingly, Ishibashi et al. (2002) have detected large amount of equol (1027 μg kg^{-1}) in a casein-based fish diet. Thus, a higher affinity of equol with cottage cheese than with whey may be explained by the equol − casein combination.

Given that equol could bind with protein, suggests that gut nitrogen flows and subsequent digestion of exogenous proteins in the gut may be influenced by equol produced in the digestive tract

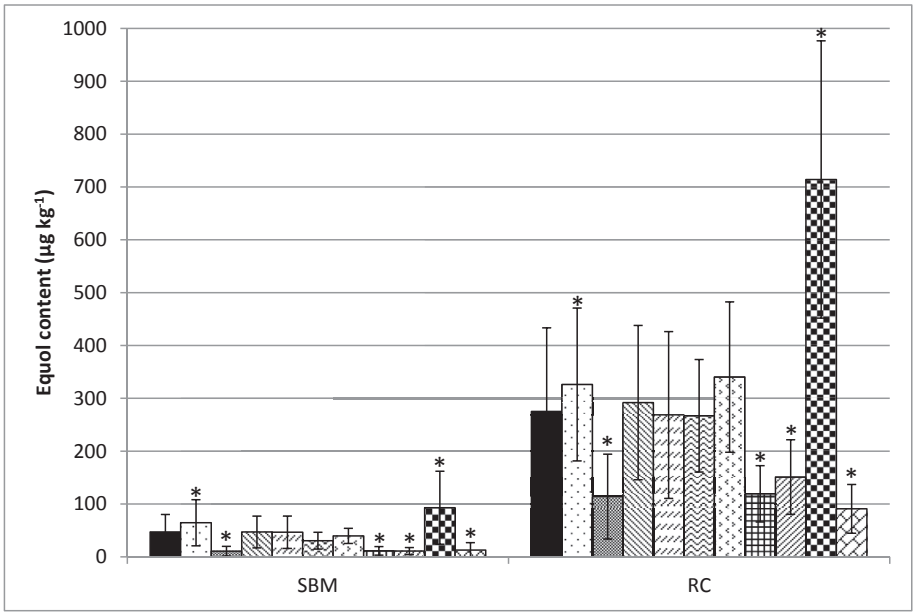

Fig. 1. Effects of process treatments on equol content in milk produced with soybean meal diet (SBM) or red clover diet (RC): fx1, raw milk; fx2, skimmed milk; fx3, cream; fx4, pasteurised milk (PA); fx5, sterilised milk (ST); fx6, yoghurt PA; fx7, yoghurt ST; fx8, kefir PA; fx9, kefir ST; fx10, cottage cheese PA; fx11, whey PA. Mean values of equol content in each dairy product are presented with standard deviation as error bars. Asterisks indicate Student's paired samples t-test comparing equol content in dairy products to the original raw milk ($P < 0.05$). Raw milk samples were only milk collected on the sixth day of each period. PA stands for products made with pasteurised milk and ST for products made with sterilised milk.

considering the fact that equol could potentially bind to proteins. To our knowledge, no study has been published on the effect of producing equol on the protein digestion in the small intestine. Kašparovská et al. (2016) have analysed the effect of isoflavones in the diet on the rumen fermentation pattern. They observed a lower individual and total VFA contents when cows fed soybean isoflavone extract and some differences in the microbial composition of the rumen which could affect the protein degradation. More studies should be conducted to confirm interactions of equol produced in rumen and intestine on digestible protein supplies and identify the mechanisms involved.

In 2013, daily per capita intake of milk and dairy products (excluding butter assumed by Kuhnle et al. (2008) to have no equol) were estimated at 647 g in Belgium, 589 g in Europe and 679 g in Northern America (FAOSTAT, 2013). Consuming milk and associated dairy products obtained with the RC diet would result in an average equol daily intake of about 182 μg for a Belgian, 166 μg for a European, and 192 μg for an American citizen. Aso (2010) and Usui et al. (2013) have shown the effect of 10 mg equol daily intake on menopausal symptoms and prevention of cardiovascular diseases in overweight or obese individuals. However, to our knowledge, the minimal amount of equol to cure or prevent different types of disease in different categories of people is not determined. Furthermore, Yee et al. (2008) reported a non-observable adverse effect level (NOAEL) of 2000 mg kg^{-1} d^{-1} of equol in rats suggesting that equol content in our milks are far from a toxic level for human, even if it is not yet established and could be different according the type of person (e.g., children versus adult, pregnant women, etc.).

4. Conclusion

This study showed that the equol content in milk was, among other factors, dependent on amount of isoflavone intake. Given that dairy cow diets can include higher amounts of red clover silage than soybean meal and given that the isoflavone content is higher in red clover silage, the latter could be considered a more efficient feedstuff for increasing the equol content in milk. Equol was not affected by heat treatments up to 120 °C or by yoghurt production processes. Equol concentrated in cottage cheese compared with original milk maybe due to an affinity of equol for proteins. The production of kefir resulted in lower equol content. However, all these products contain equol and could be potential sources for consumers who lack the digestive micro-organisms responsible for enteric equol production.

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