

Equol in milk of dairy cows is derived from forage legumes such as red clover

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The intake of isoflavones and the resulting equol contents of both plasma and milk of the same red clover-fed cows are reported for the first time in cyclic change-over design study. Cows were fed four different red clover silages and two timothy–meadow fescue silages as controls. The red clover silages contained daidzein, formononetin, biochanin A and genistein, whereas the timothy–meadow fescue silages contained no isoflavones. We found a strong association ($y = 0.071x + 2.75$, R^2 0.71) between the formononetin intake (x) and equol concentration (y) in the plasma, while the formononetin intake and milk equol concentration were weakly associated ($y = 0.0035x + 0.358$, R^2 0.20). This means that a small part of the total formononetin in the silage is secreted into milk as equol. The mean equol contents in plasma and milk of cows fed red clover silage diets were in the range of 4.6–8.4 mg/l and 458–643 µg/l, respectively, while the respective values for the control diets were in the range of 0.8–1.5 mg/l and 171–287 µg/l. We showed that shorter growing periods of red clover resulted in higher silage formononetin contents and plasma and milk equol contents, suggesting that the equol content of milk can be manipulated by varying the harvesting strategy of red clover. We conclude that milk equol is derived from the formononetin of red clover silage and that milk from red clover-fed cows can be considered as a source of equol in human nutrition.

Equol: Formononetin: Bovine milk: Isoflavones: Phyto-oestrogens: Red clover

Legume plants possess polyphenolic components such as isoflavones, which have attracted increasing interest among scientists and consumers because of their reported health benefits^(1–3). Many of the claimed health benefits have been associated with the isoflavone metabolite equol^(4–6), which is a phenolic compound found in mare's urine in 1932⁽⁷⁾. It was first believed to be oestrogenically inactive⁽⁷⁾, but later it was determined oestrogenically active giving rise to a specific breeding problem in sheep ingesting clover of high formononetin content^(8–12).

Special interest in equol in human medicine aroused in 1982, when high levels of equol were found in the urine of human adults consuming soya foods⁽¹³⁾. This discovery introduced a new aspect of the mechanism of action and effectiveness of soya to the studies of hormone-dependent diseases^(4,5). Equol possesses a high affinity for oestrogen receptor β and low affinity for oestrogen receptor α , and may thus be classified as a natural selective oestrogen receptor modulator⁽¹⁴⁾. The possible role of equol in replacing the effects of oestrogen on the bone, brain and cardiovascular and immune systems is currently under intensive investigation in human medicine and nutrition.

The human intestinal bacteria can produce equol from soya food abundant in daidzein. However, only 20–35% of the

adult human population is believed able to produce equol^(4,15,16), whereas animal populations commonly produce equol after ingesting isoflavones⁽¹⁷⁾. The isoflavones daidzein, genistein, biochanin A and formononetin are typically found in legume feeds, such as red clover (*Trifolium pratense*) silage, fed to cattle or sheep. In the rumen, biochanin A and genistein are extensively degraded to *para*-ethylphenol, whereas formononetin is demethylated to daidzein and reduced further to the isoflavan equol^(9,10,18,19).

Cow's milk is presumably the only nutriment that contains appreciable amounts of the isoflavan equol itself. In our recent study, a higher concentration of equol was detected in organically than conventionally produced commercial milk⁽²⁰⁾, which was attributed to the extensive use of forage legumes practiced on organic rather than conventional farms in Finland. Forage legumes such as red clover contain significant amounts of isoflavonoid phyto-oestrogens, while the grass silage predominantly used in conventional farming does not contain those phyto-oestrogens⁽²¹⁾. Although it seems obvious that the red clover forage fed to dairy cows is the origin of the equol in plasma and milk, to our knowledge the equol contents of both plasma and milk have not earlier been analysed simultaneously in the same experiment.

Abbreviations: RE, red clover early; RL, red clover late; RRE, regrowth of RE; RRL, regrowth of RL.

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The present study was designed to determine whether the formononetin in red clover silage fed to dairy cows is a source of equol in plasma and milk. In addition, the influence of various harvesting strategies of red clover on the equol content of plasma and milk was examined.

Experimental methods

Experimental design, animals and diets

A cyclic change-over design experiment⁽²²⁾ with twelve Finnish Ayrshire cows and six dietary treatments was conducted at the Viikki Research Farm of the University of Helsinki in 2005, as described previously⁽²³⁾. In brief, the dietary treatments consisted of six different silages offered *ad libitum* and supplemented with 9.5 kg/d of concentrate, consisting of a mixture of barley–oats (*Hordeum vulgare*–*Avena sativa*; 1:1) and rapeseed (*Brassica napus*) expellers (24%; Mildola Ltd, Kantvik, Finland). The two grass silages were prepared from primary growth of early- (June 15) or late- (June 30) cut timothy–meadow fescue (*Phleum pratense*–*Festuca pratensis*). The four red clover silages were prepared similarly from primary growth of early- (RE, July 1) or late- (RL, July 14) cut red clover and their regrowths (RRE and RRL, August 24, respectively). Since the regrowths were harvested on the same day, the growth period was shorter for the RRL than the RRE silage. The concentrate mixture was distributed to the cows in six equal doses during the day. In addition, the cows were given mineral supplements daily and they had free access to water. The study consisted of four 21-d experimental periods, with an adaptation period from day 1 to day 13 followed by a sampling period from day 14 to day 21. All feeds given and the leftovers found were weighed daily. The milk yields were recorded daily. During the last week of each period, milk samples were taken from the four consecutive milkings and pooled to form one sample per cow and period. Samples from the silages were collected daily during the last week of each period and pooled to form one sample per period. On the last day of each period, blood samples were collected twice by vacuum puncture of a jugular vein, before morning feeding and 3 h thereafter. After immediate centrifugation (4800g, 10 min), the plasma was harvested, frozen and stored at –80°C until analysis, as were the silage and milk samples. All experimental procedures were approved by the Animal Experimental Committee of the University of Helsinki, Finland, in accordance with the 1985 Use of Vertebrates for Scientific Purpose Act.

Analytical procedures

High-pressure liquid chromatography was used to analyse various isoflavonoid phyto-oestrogens in the silage, plasma and milk samples. The phyto-oestrogens daidzein, formononetin, biochanin A, genistein and coumestrol were analysed in triplicate from silage samples by a method described by Sarelli *et al.*⁽²⁴⁾. The concentrations of formononetin and its metabolites equol and demethylangolensin in the plasma samples were analysed by modification of the method described in Mustonen *et al.*⁽²¹⁾. The instrumentation used was a Liquid Chromatograph 1100 (Degasser, Japan); Binary Pump, FLD (Agilent Technologies, Germany); DAD

(Agilent Technologies); ChemStation data system (Agilent Technologies). Flavone, Sigma F 2003, was used as an internal standard. Calibration curves were established in the concentration range 1.172–75 µg/ml for equol and 0.195–12.5 µg/ml for the other compounds with correlation over 0.999. The limit of detection and the limit of quantitation were determined at signal-to-noise ratios of over 3 and 10, respectively. The results are presented in Table 1. The recovery level (93 ± 11%) was calculated with flavone. The plasma concentrations are presented as averages of the two samples collected per cow per period. The concentration of equol in milk samples was analysed using the method of Hoikkala *et al.*⁽²⁰⁾.

Calculations and statistical analysis

The daily intake of isoflavonoid phyto-oestrogens, concentrations of isoflavonoids in the plasma and milk samples as well as their daily secretion into the milk were calculated and subjected to ANOVA, using the mixed procedure of Statistical Analysis System (SAS Institute Inc., Cary, NC, USA)⁽²⁵⁾. Intake of isoflavonoid phyto-oestrogens and secretion into the milk were based on the mean values from the last 6 d of the experimental period. The statistical model used included block, period, block × period interaction and treatment as fixed factors, with the cow within the block as the random factor. The sums of squares of the treatment effects were further divided into the following pre-planned single degree-of-freedom comparisons: (1) effect of cut number of red clover silage diets (RE + RL *v.* RRE + RRL); (2) effect of growth stage of red clover silage diets (RE + RRL *v.* RL + RRE); (3) interaction between the cut number and growth stage of red clover diets; (4) effect of plant species, *i.e.* red clover *v.* grass silage diets (RE + RL *v.* grass early + grass late); (5) interaction between the plant species and growth stage of the silage diets.

Results

The isoflavonoid phyto-oestrogen contents of the experimental silages are presented in Table 2. No isoflavones were detected in the grass silages. The formononetin contents of the red clover silages were highest for the RE and RRL silages, both characterised by short growing times. The silage DM intakes were 14.3, 8.3, 10.7, 9.0, 12.8 and 11.8 kg/d (SEM 0.67 kg/d) for the grass early, grass late, RE, RL, RRE and RRL treatments, respectively. The daily milk yields were 28.6, 25.5, 28.3, 28.4, 29.1 and 29.7 kg/d (SEM 1.05 kg/d) for the respective treatments⁽²³⁾.

Table 1. The limit of detection (LOD) and the limit of quantitation (LOQ) for the analysed compounds

	LOD (µg/l)	LOQ (µg/l)
Daidzein 465 emission nm	9.9	19.8
Genistein 262 nm	39.5	79.0
Equol 310 emission nm	58.6	117.2
O-DMA 281 nm	47.9	109.6
Formononetin 465 emission nm	9.9	19.8
Biochanin-A 262 nm	39.5	79.0

O-DMA, demethylangolensin.

Table 2. Isoflavone content in the experimental silages, g/kg DM (Mean values and their standard deviations)

Plant species	Grass		Red clover							
	Primary growth		Primary growth				Regrowth			
	GE	GL	RE		RL		RRL		RRE	
	Mean	Mean	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Daidzein	0.00	0.00	0.16	0.014	0.29	0.033	0.16	0.017	0.17	0.008
Formononetin	0.00	0.00	5.15	0.298	2.95	0.549	6.47	0.499	4.31	0.181
Genistein	0.00	0.00	0.73	0.033	0.65	0.052	0.50	0.069	0.55	0.030
Biochanin A	0.00	0.00	4.37	0.243	2.52	0.416	4.65	0.326	3.23	0.219
Total*	0.00	0.00	10.41	0.555	6.40	0.962	11.78	0.788	8.26	0.281

GE, grass early; GL, grass late; RE, red clover early; RL, red clover late; RRL, regrowth of RL; RRE, regrowth of RE.

* No coumestrol was detected in the silage samples.

The intake of isoflavones as well as isoflavonoids found in the plasma and milk of the grass- or red clover silage-fed cows is presented in Table 3. The equol contents in plasma and milk were significantly higher ($P < 0.001$) for the red clover than for the grass silage-fed cows. Although the intake of formononetin (x) was strongly associated with the equol concentration in plasma ($y = 0.071x + 2.75$, $R^2 = 0.71$), it was weakly associated with the equol concentration in milk ($y = 0.0035x + 0.358$, $R^2 = 0.20$). Furthermore, the equol contents of plasma and milk were significantly higher ($P < 0.01$) for the cows fed RRE and RRL than for RE and RL silages. The equol contents in plasma were significantly higher ($P < 0.001$) for cows fed early than late cut red clover silages. No demethylangolensin was found in the milk.

Although grass silage contained no equol precursors, we found some equol in the plasma and milk of grass silage-fed cows. During the four 21-d experimental feeding periods, the cows were situated in adjacent tie-stall cubicles.

The feeding alley was partitioned individually. Despite this, snatching of silage from neighbouring cows did occur. Stealing was totally prevented during the last of the four experimental periods, and no equol was found in plasma and milk of the grass silage-fed cows during this period as presented in the Table 3.

Discussion

In the present experimental study, the equol concentration both in plasma and milk of dairy cows fed silage of known isoflavonoid content was investigated. Formononetin in the red clover silage was shown to be the source of equol in plasma and milk of dairy cows. Daidzein as a precursor of equol can contribute to the equol concentration as well. The daidzein content in silage was, however, very low and therefore its contribution to equol concentration is of minor importance in the present study. The milk equol, thus

Table 3. Isoflavonoids in the plasma and milk of grass or red clover silage fed cows

Plant species	Grass		Red clover				SEM	Statistical significances*				
	Primary growth		Primary growth		Regrowth			C1	C2	C3	C4	C5
	GE	GL	RE	RL	RRL	RRE						
Intake of isoflavonoid phyto-oestrogens (g/d)												
Daidzein	0.0	0.0	1.66	2.59	1.83	2.20	0.12	0.34	<0.001	0.02	<0.001	<0.001
Formononetin	0.0	0.0	53.5	26.8	75.6	55.7	2.58	<0.001	<0.001	0.18	<0.001	<0.001
Total†	0.0	0.0	108.3	58.1	138.0	106.6	5.29	<0.001	<0.001	<0.001	<0.001	<0.001
Concentration in plasma (mg/l)												
Equol	0.84	1.50	6.13	4.58	8.39	6.93	0.362	<0.001	<0.001	0.90	<0.001	0.22
Equol ^{4th period}	0‡	0‡	5.20	4.54	8.91	6.95						
O-DMA	0.02	0.02	0.37	0.16	0.32	0.23	0.033	0.64	<0.001	0.08	<0.001	0.002
Formononetin	0.00	0.002	0.024	0.004	0.035	0.023	0.005	0.004	0.002	0.39	0.009	0.07
Total	0.86	1.52	6.53	4.74	8.76	7.18	0.363	<0.001	<0.001	0.77	<0.001	0.12
Concentration in milk (µg/l)												
Equol	171	287	488	458	643	583	52.0	0.001	0.37	0.76	<0.001	0.39
Equol ^{4th period}	0‡	0‡	506	410	695	523						
Secretion into milk (mg/d)												
Equol	4.4	7.4	13.4	12.2	19.1	15.9	1.69	0.003	0.14	0.51	<0.001	0.56

GE, grass early; GL, grass late; RE, red clover early; RL, red clover late; RRL, regrowth of RL; RRE, regrowth of RE; O-DMA, demethylangolensin.

* Contrasts: C1, Cut number of red clover; C2, Growth stage of red clover; C3, C1 × C2 interaction; C4, Plant species i.e. red clover v. grass; C5, Plant species × growth stage interaction.

† Genistein and biochanin A are included in total intake of phytoestrogens.

‡ Possibility for stealing of red clover silage was totally prevented during the fourth experimental period.

originating from cattle feedstuffs, may contribute to human equol supply. It has been estimated that only one-third of the human population can produce equol from dietary isoflavones^(4,15,16). Setchell *et al.*⁽⁵⁾ suggested that the ability to biotransform soya isoflavones to the more potent oestrogenic equol is behind the clinical effectiveness of soya protein in cardiovascular, bone and menopausal health. Furthermore, they presented a classification of human subjects as non-equol producers and equol producers according to the level of plasma equol concentrations <10 and >20 µg/l, respectively⁽⁵⁾. Equol is predominantly a product of intestinal bacterial metabolism and can presumably not be found as such in any food other than milk. We suggest that cow's milk, with its unique nutrient content, fortified with up to 600 µg/l equol could be an interesting research theme for nutritionists.

In the present study, the average daily formononetin intake (26–75 g) of the red clover-fed cows was considerably high, as was the plasma equol concentration, (4.6–8.4 mg/l). The average equol content in milk varied between 458 and 643 µg/l. The association between plasma equol concentration and the formononetin fed to dairy cows was strong. In contrast, this strong association was not shown with intake of formononetin and equol concentration in milk, suggesting that relatively small amounts of formononetin were secreted into milk as equol. In addition, it may be that the transfer rate of isoflavonoids from feed to milk is higher at low intake than at higher intake⁽²⁶⁾. This could explain the reasonable amounts of equol found in the milk of grass silage-fed cows, which were able to snatch small amounts of red clover. In any case, considerable amounts of plasma equol are excreted in urine or faeces.

In the present experiment, the red clover-fed cows produced milk with equol concentrations of 458–643 µg/l. When King *et al.* obtained cow's milk samples from different farms in Australia, the mean equol concentration ranged from 45 to 293 µg/l. The highest equol values were found in Western Australian samples collected during spring, when the isoflavonoid-containing clover was the most dominant in pasture⁽²⁷⁾. Steinshamn *et al.*⁽²⁶⁾ fed clover silage to dairy cows without and with concentrate supplementation. With red clover feeding, daily formononetin intake ranged from 47.0 to 33.7 g/d and milk equol content 364 and 273 µg/l, respectively.

Antignac *et al.*^(28,29) investigated the occurrence of phyto-oestrogens in commercial milk samples and found equol in all analysed samples at relatively high concentrations of 14–293 µg/l. They investigated whether the type of milk sample (conventional *v.* organic agriculture) affected the phyto-oestrogen content in milk. Organic milk samples contained equol averaging 191 µg/l, whereas conventional milk samples averaged only 36 µg/l⁽²⁹⁾. In our earlier study, it was shown that Finnish commercial organic skimmed milk contained equol averaging 411 µg/l, whereas conventionally produced milk averaged 62 µg/l⁽²⁰⁾. Purup *et al.*⁽³⁰⁾ presented similar figures from Danish bulk milk samples (230 and 41 µg/l, respectively).

The equol in milk is clearly derived from the formononetin of red clover silage. Timothy–meadow fescue silages used as controls did not contain isoflavonoids. A strong association between the intake of formononetin and equol concentration in plasma was shown. Furthermore, the results suggest that

by altering the harvesting strategy, red clover silages can be manipulated to contain more formononetin, i.e. by varying the harvesting time, the formononetin content of silage was more than doubled from 3 to 6.5 g/kg in DM. When regrowth of red clover had the shortest growing period, the silage formononetin content was at the highest (6.5 g/kg in DM). Consequently, the total daily intake of formononetin and the plasma and milk equol concentrations were maximal, 76 g/d, 8.4 and 643 µg/l, respectively. The equol concentrations in plasma are well in line with earlier studies. Braden *et al.*⁽³¹⁾ fed freshly cut red clover forage to heifers and found plasma equol levels between 1 and 6 mg/l. In the studies of Lundh *et al.*⁽³²⁾, the plasma concentration of equol was determined in blood samples from dairy cattle. When the daily intake of formononetin was 13–14 g, the equol level was 2 mg/l. In an earlier study with intake of 3.5 g formononetin, the maximum equol level of 0.27 mg/l was detected 3 h after ingestion⁽³³⁾. In both studies, red clover was used as a source of isoflavones.

These results and earlier observations demonstrate that animal feeding does explain the differences in the isoflavonoid phyto-oestrogen contents in milk, the red clover forage being the main source of equol. Due to its nitrogen-fixing ability, red clover is widely used in organic agriculture, which illustrates why the phyto-oestrogen concentrations in the organic milk samples are higher than in conventionally produced milk. These results show that red clover-fed cows produce more equol in their milk, which may spark further interest in organically produced milk or milk specially produced for high equol concentration. The possible health benefits or risks associated with elevated milk equol concentration should be evaluated thoroughly as equol is classified as a natural selective oestrogen receptor modulator. Red clover-fed cows have high equol concentrations in their plasma and could be interesting material for human health-associated studies as well as studies directed to investigate cattle health.

Conclusions

This is the first study of the intake of isoflavonoids and the simultaneous measurements of equol contents of both plasma and milk of red clover silage-fed dairy cows. We showed a strong association between formononetin intake and equol concentration in plasma. The equol content in cow's milk can be as high as 600–700 µg/l with red clover silage feeding, even though it is clear that only a small part of the formononetin is secreted into milk as equol. The equol content in milk can be manipulated by varying the harvesting strategy of red clover. Shorter growing periods of red clover resulted in higher formononetin contents in the silage and equol content in the plasma and milk. Milk equol is derived from the formononetin of red clover silage and red clover-fed cows' milk can be considered as a source of equol in human nutrition.

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