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Phyto-oestrogens in herbage and milk from cows grazing white clover, red clover, lucerne or chicory-rich pastures

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A grazing experiment was carried out to study the concentration of phyto-oestrogens in herbage for cattle and in milk during two periods (May and June). Forty-eight Danish Holstein cows were divided into four groups with four treatment diets; white clover, red clover, lucerne and chicory-rich pastures. Each experimental period lasted 15 days. Herbage samples from the first day and individual milk samples from the last day of the experimental period were analysed for phyto-oestrogens using LC-MS technique. The total concentration of phyto-oestrogens was 21 399 mg/kg dry matter (DM) for red clover and 238 to 466 mg/kg DM for the other three herbages mainly due to a much higher concentration of biochanin A, formononetin and glycitein in red clover. In the milk, the total concentration of phyto-oestrogens was 253 to 397 μg/l for red clover milk and 56 to 91 μg/l in the milk from the other three treatments. This was especially due to a higher concentration of equol, daidzein and formononetin in the red clover milk. The concentration of biochanin A was significantly higher in milk from the red clover treatment in May while no differences were observed in June. Enterodiol was similar across treatments while the concentration of enterolactone was significantly lower for red clover milk compared with the other treatments. Of the tested pastures, red clover appears to have the highest concentration and to be the best source of phyto-oestrogens, especially equol, in bovine milk.

Keywords: phyto-oestrogens, bovine milk, pasture

Implications

We have documented that the milk content of specific potential health-affecting compounds may be manipulated in a predicted direction by diet composition. This implicates that milk can be designed to contain defined levels of specific compounds transferred from the diet. Examples of this are that phyto-oestrogens are transferred to milk when cows are fed high amounts of leguminous plants like clover.

Introduction

In order to increase the sale of organic milk, new specially produced organic milk types with improved taste and potential health benefits are developed. This can be done through feeding of cows with legumes or herbs containing flavour and components with possible positive impacts on health that are transferred to the milk. As for health components phyto-oestrogens are of interest. Phyto-oestrogens are a large group of naturally occurring non-steroidal plant-derived compounds with a diverse structure. Phyto-oestrogens have been shown to behave as weak oestrogen agonists/antagonists in both animals and humans (Benassayag et al., 2002) and studies in humans, animals and cell cultures suggest that dietary phyto-oestrogens may play an important role in the prevention of menopausal symptoms, osteoporosis, hormone-dependent cancers and heart disease (Kurzer and Xu, 1997). Hence, milk with an increased content of phyto-oestrogens could be of significant interest.

Cows mainly ingest phyto-oestrogens from legumes. Among the legumes, red clover has the highest total concentration of phyto-oestrogens varying from 1% to 2.5% of dry matter (DM), while the concentration of phyto-oestrogens in white clover is 0.02% to 0.06% of DM (Saloniemi et al., 1995). Both red and white clovers primarily contain isoflavones such as formononetin, genistein, daidzein and
biochanin A (Steinshamm et al., 2008). Lucerne only contains small quantities of isoflavones (Saloniemi et al., 1995). Lignans are primarily found in cereals, legumes and oilseed used in concentrates (Thompson et al., 1991), and occur in somewhat higher concentrations in white clover compared to red clover (Steinshamm et al., 2008).

In bovine milk, the concentration of the isoflavones, formononetin, biochanin A, daidzein and genistein, have been found to range from 0.1 to 7.7 μg/l, the concentration of the isoflavone metabolite equol between 45 and 364 μg/l and the lignan enterolactone between 19 and 96 μg/l (Antignac et al., 2004; Steinshamm et al., 2008; Andersen et al., 2009). In general, organic milk has a higher concentration of isoflavonoids than conventionally produced milk due to the more widespread use of leguminous plants in organic feeding (Antignac et al., 2004; Purup et al., 2005; Hoikkala et al., 2007). Steinshamm et al. (2008) found that milk from cows fed red clover silage had a significantly and several times higher concentration of isoflavones compared to milk from cows fed white clover silage. For example, the concentration of biochanin A, equal and formononetin in the milk was on average 5.0, 4.3 and 2.4 times higher, respectively, in cows fed red clover v white clover silage. In contrast, milk from cows fed white clover silage had the highest concentration of the lignans enterodiol and enterolactone (on average 1.75 and 1.37 times higher, respectively). The effects of silage type on the concentration of the individual phyto-oestrogens in the milk were related to the intake of the compound or its precursor, which implies that it is possible to influence the concentration of the phyto-oestrogens in bovine milk. However, there is limited knowledge on the concentration of phyto-oestrogens in milk from cows on different diets and at different time periods throughout the year.

In this study, the concentrations of phyto-oestrogens in milk from cows grazing red clover, white clover, lucerne or chicory-rich swards were measured during two summer months. Our hypothesis was that the milk content of phytoestrogens can be manipulated by diet composition. No literature exists on the concentration of phyto-oestrogens in chicory, but chicory was included in the study due to its flavour components. However, only the concentration of phyto-oestrogens is included in this article as the taste of the milk is reported elsewhere.

Material and methods

Pasture

A grazing study was performed in the summer 2006 at the organic research station Rugballegaard (55°52'N, 9°47'E), Denmark, with four treatment diets: white clover, red clover, lucerne and chicory-rich swards.

Swards with lucerne (Medicago sativa L., Pondus), red clover (Trifolium pratense L., Rajah), white clover (Trifolium repens L., Milo) and white clover together with chicory (Cichorium intybus L., Puna), respectively, were established in 2005 together with perennial ryegrass (Lolium perenne L.), 35% Calibre (medium tetraploid), 30% Sameba (late diploid) and 35% Tivoli (late tetraploid) in two replicate paddocks. The swards were unfertilized and irrigated at high drought stress. The paddocks were approximately 1.8 ha each (see Eriksen et al. (2007) for further details).

Before grazing, the area in each paddock was adjusted with a fence to ensure the same amount of herbage per cow in all paddocks. As little social contact as possible between the groups was ensured during allocation to the paddocks. The cows were on pasture 20 h daily, supplemented with 6.2 kg DM/cow per day (oats 82%, hay 16%, mineral mix 2%), fed twice daily after milking (0500 h and 1600 h).

Before each experimental period the cows grazed a traditional white clover/perennial ryegrass field, and from five days before the start of each period, cows were fed the experimental concentrates in the stable.

Animals and experimental design

Forty-eight lactating Holstein dairy cows, 140 ± 98 days in milk (mean ± s.d.), with milk yield 30.4 ± 5.7 kg energy corrected milk were, before each of the two periods, blocked according to milk yield and parity (1, 2 and >2), randomly within block allocated to one of the four treatments. The cows grazed the paddocks in two periods (May and June). Each period lasted 14 days with start on day 1 after morning milking and finishing on day 15 after morning milking. The cows grazed the two statistical replicates in relation to crop production alternately with one day in each paddock.

Data collection

Individual milk samples were collected on day 15 during morning milking in the experimental period. One sample from June was missing. The milk samples were frozen immediately after collection and stored at −20°C until analysis for phyto-oestrogens.

Individual milk yield was measured and concentration of fat and protein analysed three times during the period. Sward productivity was estimated indirectly in an area fenced off during the period. In the beginning and after one week of grazing the herbage mass and the botanical composition were determined in 0.5 m² samples in the grazed area and in the fenced area (Eriksen et al., 2007).

Prior to each grazing period, samples of herbage were collected by tearing off plant parts above stubble height (5 to 6 cm) by hand. This was to get a sample more representative for animal intake than the samples cut at the soil surface. Approximately 2 kg of fresh plant material was sampled. A sub-sample of 1 kg was frozen at −20°C within 15 min and used for analysis of phyto-oestrogens. Botanical composition was assumed similar in both samples. The stubble height was approximated to the lowest grazing height of the cows in the individual paddocks. Samples were collected separately in the two paddocks. The botanical composition was determined by hand separation in sub-samples, drying and weighing.

Registrations made on herd and sward productivity and botanical composition for May and June, respectively, are
shown in Table 1. Generally, the proportion of legumes and chicory was high in the mixtures. However, the proportion of lucerne in both periods was considerably lower than the other three mixtures and only 12% in June caused by a too short rest period from May. The proportion of clover, lucerne and chicory was considerably higher in May compared with June.

**Table 1** Proportion of the test species in the sward and herbage mass at the beginning of each period, and growth rate during the first week of the 2-week experimental period

<table>
<thead>
<tr>
<th>Sward type</th>
<th>Proportion of test species in the sward (% of DM)</th>
<th>Herbage mass (kg DM/ha)</th>
<th>Herbage growth rate (kg DM/ha per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>May</td>
<td>June</td>
<td>May</td>
</tr>
<tr>
<td>White clover</td>
<td>60</td>
<td>46</td>
<td>1799</td>
</tr>
<tr>
<td>Red clover</td>
<td>68</td>
<td>47</td>
<td>2383</td>
</tr>
<tr>
<td>Lucerne</td>
<td>27</td>
<td>12</td>
<td>2476</td>
</tr>
<tr>
<td>Chicory</td>
<td>72</td>
<td>52</td>
<td>1738</td>
</tr>
</tbody>
</table>

DM = dry matter.

Samples were collected from two paddocks and the botanical composition was determined by hand separation in sub-samples and weighing.
compound with the same MW, probably apigenin. Therefore, it was not possible to measure the concentration of genistein. Furthermore, the concentration of daidzein could not be measured in herbage samples.

**Statistical analysis**

All data were analysed using the GLM procedure of SAS (SAS Institute, 1999). The following statistical model was used:

\[ Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha \times \beta)_{ij} + \epsilon_{ijkl}, \]

where: \( Y \) = dependent variable, \( \mu \) = mean, \( \alpha \) = fixed effect of period \( i \) (May, June), \( \beta \) = fixed effect of diet \( j \) (white clover, red clover, lucerne, chicory); random residual variation \( \epsilon_{ijkl} \sim N(0, \sigma^2) \). For the analysis of phyto-oestrogens in herbage mixtures the fixed effect of period was excluded. The results are presented as least squares means.

**Results**

**Milk yield**

The milk yield, fat and protein concentration, reported in Table 3, show no overall effect of dietary treatment or period.

**Phyto-oestrogens in herbage samples**

The concentrations of phyto-oestrogens in the herbage are reported in Table 4. The total concentration of phyto-oestrogens was 45 to 90 times higher in red clover mixture compared to the other three herbage mixtures. In particular, the concentration of naringenin, biochanin A, formononetin and glycitein was significantly higher in red clover mixture than in the other herbage mixtures. However, although not significant, the concentration of chrysin was many times higher in the chicory mixture. The total level of phyto-oestrogens in the other three herbage mixtures was similar. In the red clover mixture both biochanin A and formononetin occurred in high concentrations compared to the other phyto-oestrogens. In the white clover mixture the by-far most quantitative phyto-oestrogen was formononetin. In the lucerne and chicory mixtures none of the phyto-oestrogens occurred in relatively high concentrations compared with the mixtures.
Phyto-oestrogens in milk samples

The concentration of phyto-oestrogens in the milk is shown in Table 5 with regard to enterolactone being the most quantitatively important phyto-oestrogen. The concentration of phyto-oestrogens was 4 to 5.6 times higher in milk from the red clover diet compared with the other treatments and more or less comparable for the other three treatments. The concentration of equol, daidzein and formononetin was 6.1 to 11.8, 2.7 to 6.6 and 2.7 to 4 times (P < 0.001) higher in milk from cows fed the red clover mixture compared with the other treatments, whereas there were no significant differences between white clover, chicory and lucerne. The concentration of biochanin A was significantly higher in milk from cows fed the red clover mixture compared to the other treatments in May, while no differences were observed in June. For naringenin and glycitein, the concentration was similar across treatments in May but significantly lower for white clover compared to the other treatments in June. The concentration of chrysin was significantly lower for white clover in June compared with the other treatments. Enterolactone was 1.3 to 2 times lower for red clover compared with the other treatments, whereas there were no significant differences between white clover, chicory and lucerne. Treatment had no effect on the concentration of enterodiol but overall the concentration was lower in June compared with May. When looking at the effect of period, the total concentration of phyto-oestrogens increased from May to June. The concentration of formononetin decreased, whereas chrysin and daidzein increased. The concentration of naringenin decreased, whereas chrysin and daidzein increased.

Table 5: Concentration (µg/l) of phyto-oestrogens in milk in relation to dietary treatments

<table>
<thead>
<tr>
<th>Phyto-oestrogen</th>
<th>May</th>
<th>June</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diet</td>
<td>Period</td>
<td>Diet × Period</td>
</tr>
<tr>
<td>Chrysin</td>
<td>0.27ab</td>
<td>0.30a</td>
<td>0.26ab</td>
</tr>
<tr>
<td>Naringenin</td>
<td>12.43ab</td>
<td>14.30c</td>
<td>14.00c</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>0.34ad</td>
<td>0.55b</td>
<td>0.25ade</td>
</tr>
<tr>
<td>Daidzein</td>
<td>0.32a</td>
<td>1.14a</td>
<td>0.42a</td>
</tr>
<tr>
<td>Equol</td>
<td>30.04a</td>
<td>355.42b</td>
<td>46.20a</td>
</tr>
<tr>
<td>Formononetin</td>
<td>1.98ae</td>
<td>6.28a</td>
<td>2.28a</td>
</tr>
<tr>
<td>Glycitein</td>
<td>3.55a</td>
<td>3.66a</td>
<td>3.68a</td>
</tr>
<tr>
<td>Enterodiol</td>
<td>7.76a</td>
<td>7.53ab</td>
<td>7.63ab</td>
</tr>
<tr>
<td>Enterolactone</td>
<td>12.47a</td>
<td>7.96b</td>
<td>16.25a</td>
</tr>
<tr>
<td>Total concentration</td>
<td>70.16a</td>
<td>397.14b</td>
<td>91.00a</td>
</tr>
</tbody>
</table>

*P-values for the effect of diet, period and their interaction are shown. The superscript symbols a, b, c, d, e, f designate significant difference (P < 0.05) between dietary treatments.
clover, but that the concentration of total isoflavones was 22% higher in fresh herbage compared to silage and hay. However, it is important to note that ensiling is a dynamic process dependent on many factors (pH, microbial population, temperature, initial herbage composition) that fluctuate, and therefore these factors potentially influence the concentration of isoflavones in silage.

**Phyto-oestrogens in milk**

Comparing the phyto-oestrogen level in this study to commercial milk samples analysed by Antignac *et al.* (2004), the concentration of enterodiol was higher for all dietary treatments, but for white clover, lucerne and chicory the concentration of biochanin, daidzein, equol and enterolactone was lower. Thus, white clover, lucerne and chicory pastures do not seem effective in increasing the concentration of phyto-oestrogens in milk. However, for red clover there was an increased concentration of formononetin and equol compared to the commercial milk samples analysed by Antignac *et al.* (2004). A high concentration of equol has also been observed in other studies with milk from cows fed red clover pastures or in organic milk production where the high concentration of equol seems to be linked to the frequent use of leguminous plants, probably specifically the use of red clover (Antignac *et al.*, 2004; Purup *et al.*, 2005; Hoikkala *et al.*, 2007).

Equol appeared to be the most abundant phyto-oestrogen in milk regardless of the dietary treatment and period. This is in accordance with previous findings (Antignac *et al.*, 2004; Hoikkala *et al.*, 2007; Steinshamn *et al.*, 2008). The very high concentration of equol in milk from especially cows grazing red clover was expected, since red clover has a high concentration of formononetin (0.8 to 11 mg/g DM) but also biochanin A (0.8 to 5 mg/g DM) depending on the part (flower, stem or leaves) and maturity of the plant, cultivar and environment (Sivesind and Seguin, 2005; Booth *et al.*, 2006). Formononetin is metabolized by microbes in the rumen via daidzein to equol which is the major isoflavone absorbed to the blood circulation after feeding red clover/grass silage, whereas biochanin A is demethylated to genistein to form mainly p-ethyl phenol (Lundh, 1995). Unfortunately, the concentration of daidzein in herbage, and genistein in herbage and milk, could not been analysed in this experiment as previously described.

The concentration of enterodiol was high while the concentration of equol, enterolactone and formononetin was low in the white clover milk compared to results obtained by Steinshamn *et al.* (2008). The red clover diet resulted in a lower concentration of enterolactone and biochanin A compared to Steinshamn *et al.* (2008). For the lucerne diet, the concentration of enterodiol and equol was much higher in the present study than observed in milk from cows fed lucerne silage (Andersen *et al.*, 2009). In contrast, this study found a lower concentration of enterolactone and a slightly lower concentration of biochanin A and daidzein. The variation between experiments in the concentration of phyto-oestrogen in the milk is likely to reflect initial differences in the concentration of phyto-oestrogens in the diet but could also be due to differences in the metabolism between pasture and silage.

The concentration of naringenin and chrysion in feedstuffs and milk has not been reported before. Similarly, there are no previous studies on phyto-oestrogens in chicory. The concentration of glycitein in red clover has been reported by Tsao *et al.* (2006) who found similar levels as those found in this study. The concentration of glycitein in the other herbagas and milk has not been reported previously.

Compared to sources rich in phyto-oestrogens like soy milk (5 to 10 mg isoflavones/kg) and tofu (13.5 to 67 mg isoflavones/kg) (Committee on Toxicity of Chemicals in Food, 2003), the concentration of phyto-oestrogens in bovine milk is low. Nevertheless, the mixture of many phyto-oestrogens could have an additive or synergistic effect and thus a biological effect. For instance, a recent experiment showed that soy extract is more potent than genistein, the most potent phyto-oestrogen in soy, in inhibiting tumour growth. This is presumably due to the synergistic effect of the various bioactive components in the soy extract (Kim *et al.*, 2008). Future studies will have to investigate if there is a biological effect of phyto-oestrogens in bovine milk. This study verifies that it is possible to affect the concentration of phyto-oestrogens in the milk through the feeding and that red clover diets seem to be the most effective way of increasing the concentration of phyto-oestrogens in bovine milk.

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**References**


Phyto-oestrogens in herbage and milk


