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Seasonal variation in contents of phytoestrogens in Danish dairy milk lines of different farm management systems

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ABSTRACT

Farm management systems and seasonal variation are suggested to affect the concentrations of phytoestrogens in milk. The present study analyses the concentrations of phytoestrogens and their relative composition in distinct milk lines from six Danish dairies representing three management systems (conventional, organic and biodynamic) in five sampling periods (P1–P5) over one year. A highly selective and sensitive liquid chromatography-triple quadrupole mass spectrometry method was applied to determine absolute concentrations of phytoestrogens in the different milk lines over the period. Biodynamic dairy milk lines contained higher levels of most isoflavones compared with milk from conventional systems. This may be explained by the differences in feeding strategies and, more specifically, botanical composition of feed. In relation to season, the concentration of the mammalian derived isoflavone, equol, was highest during the indoor period in biodynamic milk. Compared with the isoflavones, concentrations of lignans were less affected by farm management system.

1. Introduction

It is a relatively new field to identify and quantify milk phytoestrogens, especially in absolute concentrations, i.e., ng mL^{-1}. Phytoestrogens have structural similarities to mammalian estrogens and may bind to estrogen receptors and thereby potentially act like estrogens or anti-estrogens in humans, but their potential nutritional and health related effects on humans are still being debated (Domínguez-López, Yago-Aragón, Salas-Huetos, Tresserra-Rimbau, & Hurtado-Barroso, 2020; Morand & Tomás-Barberán, 2019; Nielsen, Nørgaard, Purup, Fretté, & Bonefeld-Jørgensen, 2009; Nørskov, 2021; Purup & Nielsen, 2012). Hence, nutritional recommendations are not yet available.

Phytoestrogens comprise a larger group of plant secondary metabolites naturally occurring in several plants and seeds, including those supplemented to ruminants from where they are transferred into the milk. In addition to being transferred from feed, some milk phytoestrogens are of mammalian origin through bioconversion of plant-derived compounds (Heinonen et al., 2001; Njåstad et al., 2014). Among the phytoestrogens in feed, the major groups are isoflavones (formerly called flavonoids), lignans and coumestans. The isoflavones formononetin, daidzein, biochanin A, glycitein, genistein and prunetin are directly derived from plants and a fraction of them transferred to milk via the feed (Njåstad et al., 2014; Nørskov, Givens, Purup, & Stergiadis, 2019). Most of the isoflavones ingested (biochanin A, genistein, daidzein and formononetin) are, however, further metabolised into the mammalian isoflavone, equol, which in cows are then transferred to milk. The equol concentration has been reported to be especially high in milk from cows fed diets containing high amounts of red clover (Njåstad et al., 2014; Steinshamn, Purup, Thuen, & Hansen-Møller, 2008). Oppositely, white clover contains more lignans and coumestans compared with red clover (Adler et al., 2014; Andersen, Weisbjerg, Hansen-Mller, & Sejrsen, 2009b; Mustonen et al., 2009; Nørskov et al., 2019). The lignans include both the plant derived secoisolariciresinol, matairesinol and lariciresinol, and the mammalian enterolactone and enterodiol. The feed derived phytoestrogens are conjugated with glucuronic or sulfonic acids through the digestion process in the rumen, which facilitates their transfer into the blood, and thereby into milk (Branca & Lorenzetti, 2005; Viggiani, Polimeno, Di Leo, & Barone, 2019); an effect that however according to a recent report, may reduce the penetration abilities of the plant derived lignans to milk (Zhuang et al., 2021).

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Milk phytoestrogens are affected by variation in feed through the composition of plant material in the diet and therefore different farm management systems (conventional, organic, biodynamic) with different feeding strategies can influence the level and composition of phytoestrogens in tank milk received at the dairies (Adler et al., 2014; Höjer et al., 2012; Nørskov et al., 2019). Depending on subsequent mixing of these different tank milk loads from individual farms with other car loads these feeding and management derived variances may manifest into milk lines and derived dairy products. Differences in phytoestrogen contents between conventional and organic milk have been investigated in Finland, Norway, Denmark and in the UK (Adler, Purup, Hansen-Møller, Thuen, & Steinshamn, 2015; Hoikkala et al., 2007; Nørskov et al., 2019; Purup et al., 2005). Hoikkala et al. (2007) compared phytoestrogen composition and concentrations in different types of commercial drinking milk, i.e., after low pasteurisation. They reported more than 6-fold higher equol concentrations in organic drinking milk compared with conventional. Also, they found traces of the plant derived isoflavones daidzein and formononetin in the organic, but not conventional milks (Hoikkala et al., 2007). In addition, milk from short-term grass management contained even higher levels of equol compared with organic production (Adler et al., 2015).

In relation to management-related effects of milk phytoestrogen contents, it is interesting to include milk from biodynamic management systems. Biodynamic farming is a practice that builds on top of organic, being an agricultural philosophy developed by Rudolph Steiner in Switzerland in 1924. These are characterised as being low-input systems focusing on a holistic approach under strict regulations, integrating composting, soil maintenance, manure cycling, plant growth and livestock care (including fertility) in an interrelated practice (Baars, Wohlers, Rohrer, Lorkowski, & Jahreis, 2019; Brock, Geier, Greiner, Olbrich-Majer, & Fritz, 2019; also see: Biodynamic Federation Demeter, https://demeter.net/about/).

In line with effects of feeding and farm management systems, influence of indoor/outdoor periods, and thereby seasonal effects on bovine milk phytoestrogen contents have been reported. A recent study on retail milk from the UK reported significant differences in phytoestrogen concentrations relative to season (Nørskov et al., 2019), with lignans having higher concentrations during Summer, while concentrations of isoflavones (both individual and in total) were higher during Winter.

The present study investigates variation in contents of phytoestrogens in a total of 11 specific Danish dairy milk lines of conventional, organic or biodynamic farm management systems over one year of seasonal change. The aim of this work was to evaluate variations observable in the distinct dairy milk lines by determining absolute levels of isoflavones (formononetin, daidzein, genistein and equol) and lignans (secoisolariciresinol, matairesinol, lariciresinol, enterolactone and enterodiol) using liquid chromatography-triple quadrupole mass spectrometry (LC-Triple Q/MS), according to an earlier developed method as applied for retail milk (Nørskov et al., 2019), to decipher levels of variation attributable to farm management system.

2. Materials and methods

2.1. Sampling of dairy milk lines

A total of 103 milk samples representing distinct dairy milk lines from 6 different Danish dairies were collected once a month over a one year period from June 2019–June 2020. There is a few missing samplings due to various reasons related to various practical issues at the dairies. The milk was sampled directly from dairy silos or tanks as raw milk, and was not standardised, homogenised or pasteurised. A total of 11 distinct milk lines representing the farm management systems; conventional (C1–C3), organic (O1–O6) and biodynamic (B1–B2) (Table 1). The milk lines further represented variances in line complexity, depending on whether the silo from where the dairy milk line originated of comprised milk from a single farm tank or milk combined from several farms, as indicated in Table 1. Furthermore, the 11 distinct dairy lines represented different breeds (Danish Holstein, Danish Jersey, cross-breed or mixes of these) and feeding practices, as characterised by mixed ration, exclusive grass feeding (all year) or hay feeding (all year). The samples were transported cold from the dairies to Aarhus University, Department of Food Science for aliquoting and further analyses. Due to a longer distance to some dairies, some samples were frozen at −45 °C until being transported to Aarhus University. Fat and protein content was determined on whole milk samples as triplicates using infra-red spectroscopy (Milkoscan™ FT2, Foss Analytical, Hillerød, Denmark).

After skimming (centrifugation for 30 min at 2671×g at 4 °C) they were all frozen in at −80 °C, transferred to and kept at −20 °C until further analysis.

Table 1
The 11 distinct dairy milk lines included in the study, representing farm management systems (conventional C1-3, organic O1-6 and biodynamic B1-2), milk line complexity, breed, feeding and number of samples taken from each milk stream over the one year period.[a]

Production type	Dairy milk stream	Abb.	Management	Milk line complexity	Breed	Feeding	Geographic area	Samples (no)
Conventional		C						30
	Conventional1	C1	Conventional	Silo	Mix	Mixed	South-Jutland	11
	Conventional2	C2	Conventional	Silo	Mix	Mixed	West-Jutland	12
	Conventional3	C3	Conventional	Silo	Mix	Mixed	Island east	7
Organic		O						55
	Organic1	O1	Organic	Silo	Mix	Mixed	South-Jutland	11
	Organic2	O2	Organic	Silo	Mix	Mixed	West-Jutland	12
	Organic3	O3	Organic	Farm tank	Mix	Mixed	Island east	8
	Organic Grass1	O4	Organic	Farm tank	Holstein	Grass	Zealand	8
	Organic Grass2	O5	Organic	Farm tank	Cross-breed	Grass	Zealand	8
	Organic Grass3	O6	Organic	Farm tank	Cross-breed	Grass	Mid-Jutland	8
Biodynamic		B						18
	Biodynamic	B1	Biodynamic	Silo	Mix	Mixed	South-Jutland	8
	Biodynamic	B2	Biodynamic	Farm tank	Cross-breed	Hay	South-Jutland	10

[a] Milk line complexity indicates whether the dairy milk represents silo milk from several farms or from only one single farm tank. Mix breed indicates herds with a mixture of breeds (combinations of Danish Holstein, Danish Jersey, Danish Red or cross-bred), Cross-breed represents herds of cross-bred Danish Holstein and Danish Jersey with monitored cross-breeding programs. Feeding indicates feeding practice, being mixed ration, grass or hay feeding.

2.2. Quantification of phytoestrogens by LC-Triple Q/MS

In total, 10 individual phytoestrogens (formononetin, daidzein, genistein, naringenin, equol, matairesinol, lariciresinol, secoisolariciresinol, enterolactone and enterodiol) were analysed in the 103 seasonal dairy line samples. The frozen skim milk samples were thawed and handled as described by Nørskov et al. (2019). All analyses were conducted in November 2020. The quantification of phytoestrogens in milk was performed using LC-Triple Q/MS using internal standards of the analysed phytoestrogens according to the method described by Nørskov et al. (2019).

2.3. Dairy lines, groupings and statistical analyses

Due to the limited number of samples, the sampling months were grouped into five periods (P1, June, July, August 2019; P2, September, October 2019; P3, November, December 2019, January 2020; P4, February, March 2020; P5, April, May, June 2020), based upon seasons and associated indoor/outdoor periods. According to Danish regulations for organic (including biodynamic) farming, cows should have access to outdoor areas in the summer period from April 15th to November 1st, while conventional farms are not obliged to have their cows outside during this period, although some do [see Økodag (Eco Day), Ökologisk Landsforening https://xn–kodag-uua.dk/om-oekodag/]. This represents a major difference between organic and biodynamic systems on one side and conventional on the other. Therefore, seasonal periods were here assigned in accordance with this, having periods 1, 2 and 5 being outdoor and periods 3 and 4 indoor. It is important here to point out, that indoor and outdoor seasons are not 100% reflecting feeding as the proportion of, e.g., fresh grass or silage may vary from farm to farm.

Cow breeds were not included as a factor in the present analysis as most of the milk was from mixed or cross-bred herds; however, information on breed is included in Table 1. Management system, but not individual feeding strategies, as the number of samples within each group were very limited, were included in the statistical analyses. Data were thus analysed for statistically significance by a linear mixed model conducted in R (version 4.0.0; http://www.r-project.org) to test for the influence of management system and sampling period, as well as their interaction, on the concentrations of phytoestrogens. Response variables were concentration (ng mL^{-1}) of the individual phytoestrogens as well as the sum of different sub-groupings, like e.g. plant isoflavones. Fixed factors were management system (conventional, organic or biodynamic) and period (P1–P5). Individual dairy was set as random variable. The analysis of variance (ANOVA) was used to compare data for each of the phytoestrogens.

3. Results

3.1. Overall variation in levels of different types and groups of phytoestrogens

Concentration of the isoflavones showed relatively large variations, with CV% varying from 57.87 to 134.91 (Table 2). In general, the mammalian isoflavone, equol, and the mammalian lignan, enterolactone, were detected in higher concentrations compared with their plant derived precursors. The mean concentration of equol was 213.65 ng mL^{-1}, approximately 20-fold higher than the concentration of the plant isoflavones (mean 10.26 ng mL^{-1}), being its precursors. The mammalian lignans, enterolactone and enterodiol, were found in 14-fold higher concentrations (mean 69.58 ng mL^{-1}) compared with the plant lignans with average value of 0.49 ng mL^{-1}. Overall, the total concentrations of isoflavones (mean 223.91 μg mL^{-1}) were approximately 3-fold higher than the lignans (70.07 μg mL^{-1}).

Table 2
Mean levels, ranges and coefficient of variation of individual phytoestrogens as determined by LC-Triple Q/MS across all 103 dairy line skim milk samples.[a]

Phytoestrogen	Mean ± SD (ng mL^{-1})	Range (ng mL^{-1})	CV[a] (%)
Plant isoflavone			
Formononetin	4.44 ± 5.99	0.25–44.73	134.91
Daidzein	3.20 ± 2.46	0.60–14.93	76.77
Genistein	1.88 ± 1.09	0.02–5.45	57.87
Naringenin	0.73 ± 0.47	0.20–3.19	64.26
Sum of plant isoflavone	10.26 ± 9.22	2.26–65.03	89.90
Mammalian isoflavone			
Equol	213.65 ± 162.64	18.81–699.30	76.12
Sum of isoflavone	223.91 ± 169.27	25.86–727.67	75.60
Plant lignans			
Matairesinol	0.17 ± 0.13	0.00–0.63	79.71
Lariciresinol	0.19 ± 0.14	0.00–0.78	73.45
Secoisolariciresinol	0.14 ± 0.11	0.00–0.79	83.57
Sum of plant lignans	0.49 ± 0.28	0.11–1.32	57.81
Mammalian lignans			
Enterolactone	69.18 ± 63.81	21.70–505.61	92.25
Enterodiol	0.41 ± 0.21	0.14–1.53	52.70
Sum of mammalian lignans	69.58 ± 63.94	21.84–507.46	91.89
Sum of lignans	70.07 ± 64.05	22.12–508.46	91.40
Other milk compounds			
Fat (g 100 g^{-1})	4.37 ± 0.38	3.49–5.57	8.58
Protein (g 100 g^{-1})	3.57 ± 0.16	3.22–4.00	4.49

[a] CV (%) represents the coefficient of variation for each trait.

3.2. Concentrations of phytoestrogens in dairy milk lines relative to farm management system and seasonal period

The plant isoflavones, daidzein and genistein varied significantly among management systems, with levels in biodynamic milk being significantly higher than in both conventional and organic. The mean concentration of the sum of the four plant isoflavones (daidzein, formononetin, genistein and naringenin) was 5.26 ng mL^{-1} in conventional milk, 10.26 ng mL^{-1} in organic and 19.09 ng mL^{-1} in biodynamic milk (Table 3). The plant isoflavones daidzein, genistein and naringenin were all affected by sampling period (Table 3). In biodynamic dairy lines, the concentration of daidzein was highest in P2 (outdoor), while the concentration of genistein was highest in P4 (indoor), and naringenin highest at P3 (indoor) (Fig. 1). The concentration of the mammalian isoflavone, equol, varied significantly among all three management systems. It was lowest (102.6 ng mL^{-1}) in conventional milk and highest (412.6 ng mL^{-1}) in biodynamic milk (Table 3). These differences in equol concentrations were also reflected in the sum of isoflavones. The seasonal effect of organic and biodynamic farming compared with conventional on milk equol and total isoflavones concentrations was more pronounced in the winter than summer as indicated by the farming system by period interaction (P-value <0.01) (Table 3). This also indicates that neither management system nor seasonal periods as variables can be interpreted independently.

The plant lignans were found not to vary significantly among the management systems in the present study. Matairesinol showed significant seasonal variation, with the lowest concentrations in P3 (indoor) for the biodynamic milk and in P4 (indoor) for both conventional and organic milk (Fig. 2). Furthermore, the level of matairesinol from biodynamic milk expressed a more pronounced effect of season compared with organic and conventional milk. The matairesinol concentration in biodynamic milk was highest in the early summer outdoor periods, P1 and P5, and lowest in the indoor period, P3. The secoisolariciresinol concentration was not affected by management system or seasonal period individually, but it

Table 3
Milk phytoestrogen concentration in the analysed dairy milk lines according to farm management system.[a]

Phytoestrogen	Concentration (ng mL^{-1}; mean ± SE) by farm management system			ANOVA *P*-values		
	Conventional (n = 30)	Organic (n = 55)	Biodynamic (n = 18)	Management	Period	Management × period
Plant isoflavone						
Formononetin	1.67 ± 1.71	4.41 ± 1.22	9.54 ± 2.14	0.060	0.100	0.158
Daidzein	1.70 ± 0.58^{a}	3.20 ± 0.41^{a}	5.81 ± 0.73^{b}	0.008	0.035	0.055
Genistein	1.30 ± 0.19^{a}	1.87 ± 0.14^{a}	2.92 ± 0.25^{b}	0.004	0.01	0.316
Naringenin	0.57 ± 0.12	0.78 ± 0.09	0.82 ± 0.15	0.348	0.003	0.144
Sum of plant isoflavones	5.26 ± 2.39^{a}	10.26 ± 1.71^{ab}	19.09 ± 3.00^{b}	0.022	0.064	0.107
Mammalian isoflavones						
Equol	102.6 ± 29.65^{a}	220.3 ± 21.23^{b}	412.6 ± 37.46^{c}	<0.001	<0.001	<0.01
Sum of isoflavones	107.8 ± 31.09^{a}	230.6 ± 22.26^{a}	431.71 ± 39.27^{c}	<0.001	<0.001	<0.01
Plant lignans						
Matairesinol	0.10 ± 0.05	0.16 ± 0.04	0.27 ± 0.06	0.174	0.011	0.019
Lariciresinol	0.17 ± 0.04	0.18 ± 0.03	0.24 ± 0.05	0.479	0.219	0.076
Secoisolariciresinol	0.11 ± 0.02	0.13 ± 0.01	0.17 ± 0.03	0.307	0.241	0.015
Sum of plant lignans	0.39 ± 0.08	0.47 ± 0.06	0.67 ± 0.11	0.164	0.146	0.013
Mammalian lignans						
Enterolactone	62.39 ± 18.57	73.93 ± 13.25	60.93 ± 23.26	0.830	0.121	0.345
Enterodiol	0.35 ± 0.09	0.40 ± 0.06	0.49 ± 0.11	0.583	0.002	0.023
Sum of mammalian lignans	62.74 ± 18.61	74.32 ± 13.28	61.42 ± 23.32	0.831	0.120	0.342
Sum of lignans	62.74 ± 18.61	74.33 ± 13.28	61.42 ± 23.32	0.833	0.119	0.336
Other milk compounds						
Fat (g 100 g^{-1})	4.25 ± 0.14	4.37 ± 0.10	4.74 ± 0.17	0.123	0.022	0.006
Protein (g 100 g^{-1})	3.58 ± 0.03^{a}	3.50 ± 0.02^{a}	3.78 ± 0.37^{b}	<0.001	0.002	0.709

[a] Significant differences between farm management systems are marked by different superscript letters within rows ($P < 0.05$); these are obtained by a comparison of Least Squares Means (LS Means) using a Tukey's test. *P*-values from the linear mixed model for the main effects of management system and period and their interaction are included.

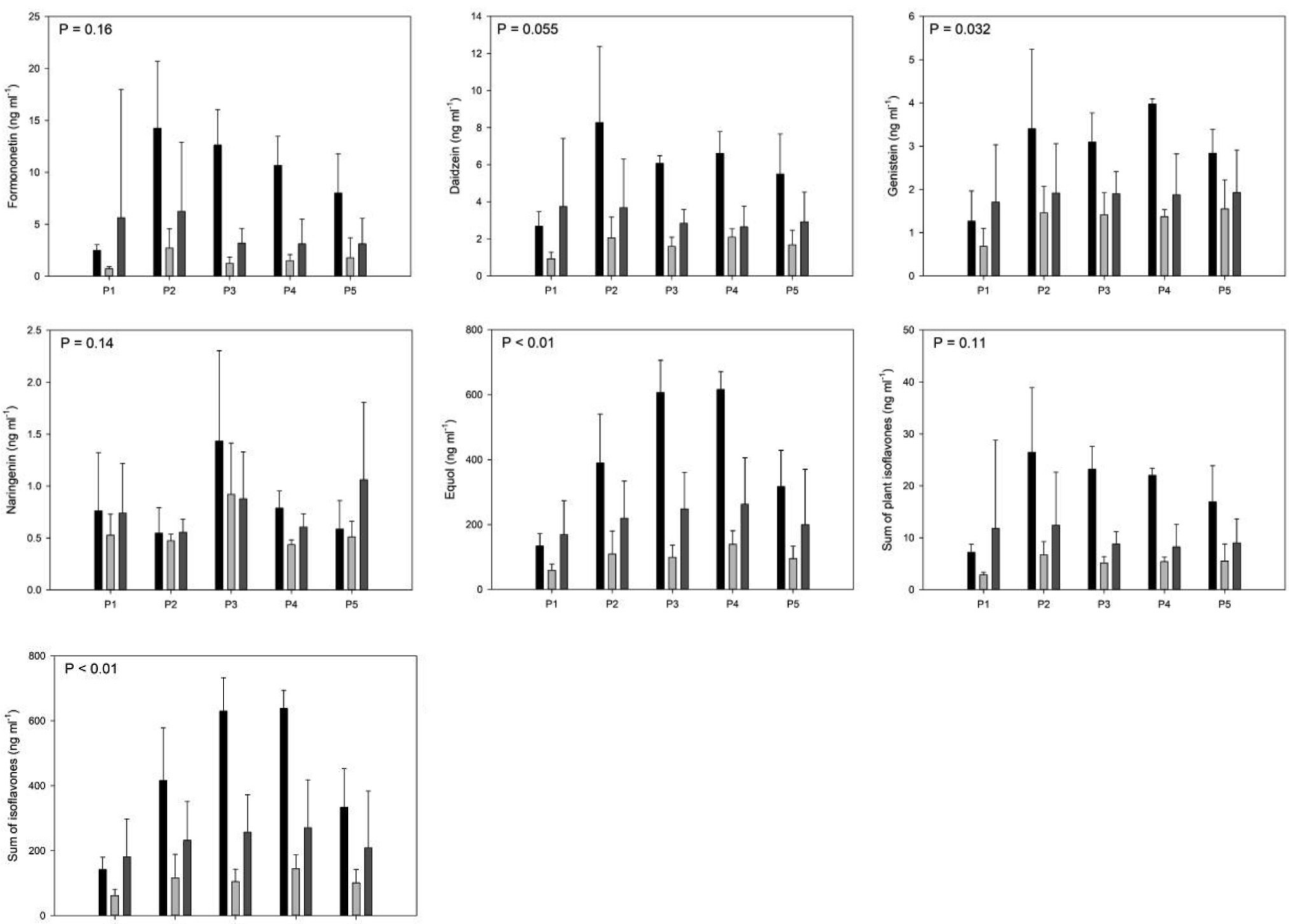

Fig. 1. The farm management (■, biodynamic; ■, conventional; ■, organic) and seasonal effects on individual and summed isoflavone levels in milk. *P*-values show the effect of the farm management × period interaction. The X-axis represents the seasons with sampling months divided into periods: P1, June 2019, July, August 2019; P2, September, October 2019; P3, November, December 2019, January 2020; P4, February, March 2020; P5, April, May, June 2020.

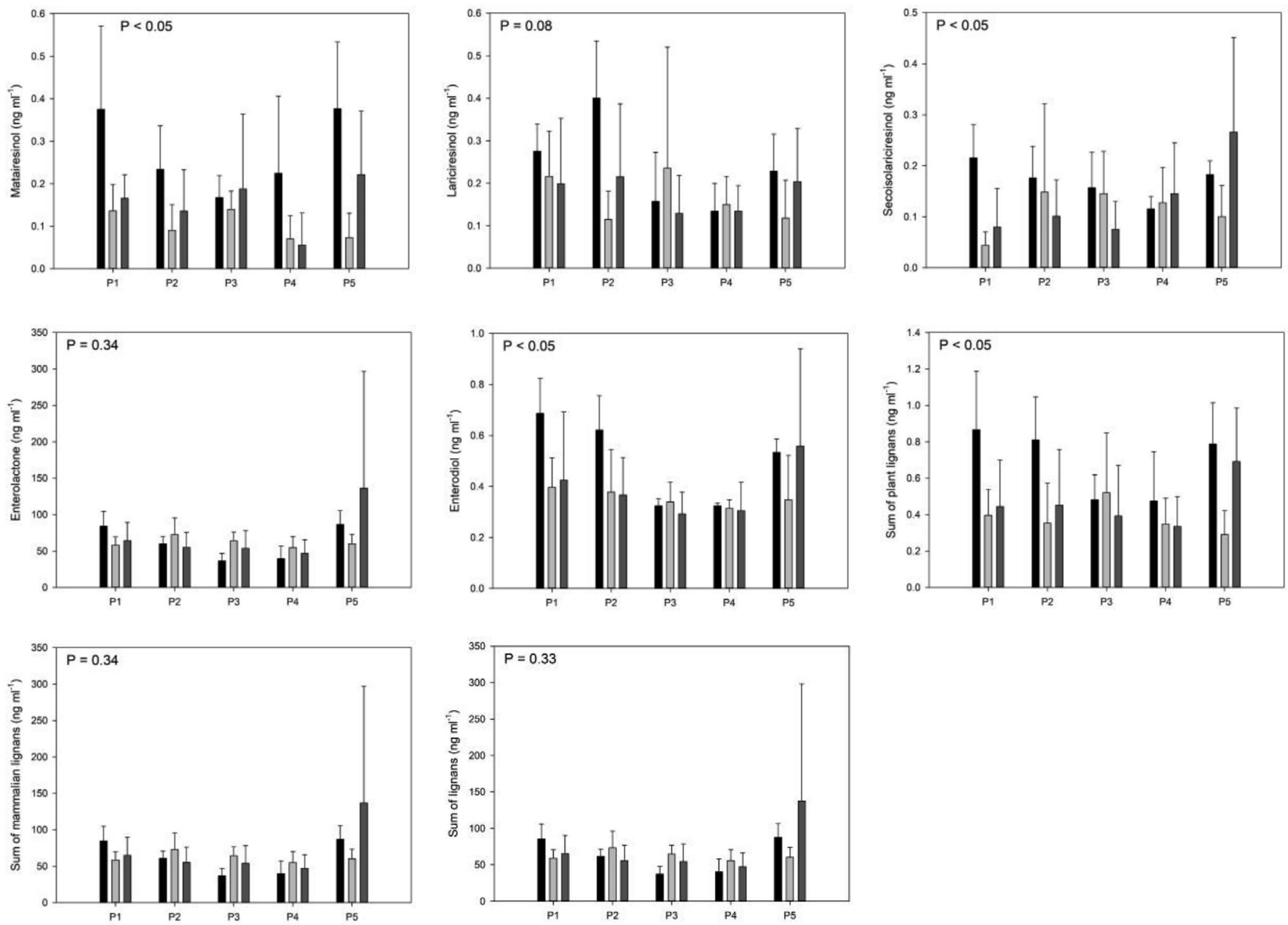

Fig. 2. The farm management (■, biodynamic; ■, conventional; ■, organic) and seasonal effects on individual and summed lignan levels in milk. *P*-values show the effect of the farm management × period interaction. The X-axis represents the seasons with sampling months divided into periods: P1, June 2019, July, August 2019; P2, September, October 2019; P3, November, December 2019, January 2020; P4, February, March 2020; P5, April, May, June 2020).

showed interaction effect. The concentration of secoisolariciresinol in biodynamic milk was highest in P1, P2 and P5 (outdoor) and lowest in period P3 (indoor) and P4 (indoor). The concentration in organic milk was lowest in P1 and increasing gradually to the highest level in P5. The concentration in the conventional milk was lowest in P1 and P5 and highest in P2, P3 and P4. The mammalian lignan, enterodiol, was also significantly affected by period, as well as the interaction of management system and period. Biodynamic milk had the highest concentration of enterodiol in P1, P2 and P5 (outdoor season).

4. Discussion

4.1. Overall concentrations of phytoestrogens in Danish dairy milk

The results showed considerable variation in content of distinct phytoestrogens in dairy milk lines from the different farm management systems and in some cases, also with seasonal variation. In line with previous studies, the mammalian equol was found in higher concentrations compared with the plant isoflavones. As this compound is the end-product from mammalian metabolism of the plant isoflavones ingested through the feed, e.g., forage from preserved or grazed legumes, grass and clover, and/or soy in compound feed (Hoikkala et al., 2007), this was expected, and in line with previous studies (Adler et al., 2014, 2015; Andersen et al., 2009a; Hoikkala et al., 2007; Njåstad et al., 2014; Nørskov et al., 2019). Likewise, the concentration of the mammalian lignan, enterolactone, was also found in higher concentrations compared with the plant lignans, also presumably reflecting enterolactone being the end-product of mammalian plant lignan metabolism. The enterolactone levels found in present study were lower than the values reported in some studies (Adler et al., 2015; Höjer et al., 2012), but also higher than the levels found in other studies (Njåstad et al., 2014; Steinshamn et al., 2008). However, here the enterolactone levels found were comparable with those reported by Nørskov et al. (2019) using the same analytical method, which according to Bláhová, Kohoutek, Procházková, Prudíková, and Bláha (2016) may be of great importance, as analytical method determining phytoestrogen concentration is highly dependent on the extraction due to potential overestimation of the concentrations of some of the phytoestrogens (Bláhová et al., 2016). The relatively high CV% (Table 2) and standard deviations (Figs. 1 and 2) indicate substantial heterogeneity in phytoestrogen levels among samples. The intermediate metabolite, enterodiol, was found in much lower concentrations than enterolactone, which is also in line with other studies (Adler et al., 2015; Höjer et al., 2012; Njåstad et al., 2014; Nørskov et al., 2019). This may suggest that most of the enterodiol has been converted from the plant lignans to enterolactone by colon microbes prior to secretion into the milk (Côrtes et al., 2008; Njåstad et al., 2014).

The plant lignans matairesinol, lariciresinol and secoisolariciresinol were overall found at levels comparable with Nørskov et al.

(2019), only with lariciresinol observed in slightly lower concentrations here. Others have reported higher levels of the plant lignans. Especially the levels of secoisolariciresinol have been reported in higher levels compared with present findings (Adler et al., 2015; Höjer et al., 2012; Njåstad et al., 2014). The plant isoflavone levels have also been characterised by great variation among studies. Our findings of the plant isoflavones are higher than the ones reported by Nørskov et al. (2019), but very similar to the ones presented by Adler et al. (2015) and by Njåstad et al. (2014).

The variation in phytoestrogen concentrations among different studies can be partly explained by the differences in origin of milk samples, as proposed in Nørskov et al. (2019). The present study was performed on raw, non-pasteurised milk lines from dairy silo milk collected, whereas other studies were performed on commercial retail milk (Nørskov et al., 2019) or on milk from individual cows (Höjer et al., 2012; Njåstad et al., 2014; Steinshamn et al., 2008). Additionally, cow genetics including breed, lactation stage, as well as further differences in diets and other management practices are other factors potentially influencing the concentration and composition of phytoestrogens as shown here and by Nørskov et al. (2019).

4.2. Phytoestrogen concentrations and composition in dairy milk lines relative to farm management systems and seasonal periods

In the present study, the plant isoflavones were, in general, observed at lowest concentrations in conventional and with highest levels in biodynamic milk (Table 3). These findings are in line with previous studies of bulk and retail milk (Adler et al., 2015; Antignac, Cariou, Le Bizec, & André, 2004; Hoikkala et al., 2007; Nørskov et al., 2019). Adler et al. (2015) compared both organic and conventional bulk silo milk with either short- or long-term grassland management and also found significant differences between organic short- and long-term management, with higher levels of isoflavones in the organic short-term grassland management. This was directly linked to the differences in the botanical composition and specifically the red clover content in the herbage (Adler et al., 2015). Of the plant isoflavones, in the present study, significant seasonal effects were observed for daidzein, genistein and naringenin. However, none of them showed a clear and consistent variation pattern relative to indoor/outdoor periods as observed in other studies (Adler et al., 2015; Nørskov et al., 2019), though the concentration in P3 (indoor) and P4 (indoor) were higher than in P1 (outdoor, early summer) and P5 (outdoor, early summer), suggesting a trend of the concentration to be related to indoor/outdoor periods.

The equol concentrations detected in present study were significantly affected by both management systems, seasonal periods as well as their interactions, with especially the biodynamic dairy milk lines being most affected by season. The study by Nørskov et al. (2019) included milk from free-range farms beside milk from conventional and organic farms. Interestingly, the level of isoflavones (including equol) in that study did not vary significantly between free-range and conventional milk. A possible explanation may be the similarities in diets in terms of forage to concentrate ratio and the forage species used (Nørskov et al., 2019).

The overall level of equol in biodynamic milk in the present study (420 ng mL^{-1}) is similar to levels found earlier in organic retail milk in the UK (411 ng mL^{-1}) (Nørskov et al., 2019). As biodynamic farming is an add-on to organic farming, some of the same practices apply. However, in addition, biodynamic farms have more regulations, especially regarding crops providing feed to the cows. The biodynamic dairy milk lines in the present study are from dairies with a very diverse mix of different plants naturally growing on the areas used for grazing. Detailed information on the composition of the feed was, however, not available, but it has earlier been argued that a high level of equol is related to high intakes of the precursors, formononetin and daidzein (Adler et al., 2015; Mustonen et al., 2009). Several studies have found high levels of formononetin in red clover, suggesting that this is related to a higher concentration of equol in milk. Hence, a higher proportion of especially red clover in the diet is related to a higher level of equol in the milk (Adler et al., 2014; Andersen et al., 2009a; Steinshamn et al., 2008). Additionally, including concentrate in the diet is argued to be associated with a decrease in isoflavones and, hence, equol concentration, as concentrate supplementation reduces the silage intake, while increasing the levels of lignans from grains. Especially, the plant lignans secoisolariciresinol and matairesinol are found in legumes, cereals and oilseeds that are further used in concentrates (Steinshamn et al., 2008; Thompson, Robb, Serraino, & Cheung, 1991). As organic (including biodynamic) cows in general have a lower concentrate intake than conventional, this may also be a part of the explanation of the great differences in equol concentrations related to management system in present and in previous studies (Adler et al., 2015; Njåstad et al., 2014; Nørskov et al., 2019; Steinshamn et al., 2008).

Equol was observed in higher concentrations during the indoor periods. This was also reflected in the sum of isoflavones and is in line with previous studies (Adler et al., 2015; Nørskov et al., 2019). A possible explanation for this trend could be the relatively high proportion of clover in the silages compared with pasture. Clover-grass and lucerne are among the most common crops used for silage in Denmark as well as in other Northern European countries (Dehghani, Weisbjerg, Hvelplund, & Kristensen, 2012; Hansen et al., 2021). As the harvesting of silage swards is usually done when the biomass of clover is relatively high (Höjer et al., 2012; Nørskov et al., 2019; Steinshamn et al., 2008), it has been argued, that the time of harvesting clover for silage is important for the stem:leaf ratio, and as the concentration of isoflavones, in general, is higher in leaves compared with stems (Tsao, Papadopoulos, Yang, Young, & Mcrae, 2006), this may be one of the explanations for higher isoflavone levels during the indoor period (Nørskov et al., 2019). Another important factor to take into consideration is the botanical composition of the material used for silage, as the individual phytoestrogens and their metabolites are highly dependent of the different grass, clover and other plant species (Steinshamn et al., 2008).

In general, the concentrations of lignans were not varying significantly among management systems. This is in contrast with other studies, where concentrations of the plant lignan secoisolariciresinol were observed higher in organic milk than in conventional milk (Adler et al., 2015; Nørskov et al., 2019), though Nørskov et al. (2015) also argued that the sum of lignans did not vary with management system.

In the present study, matairesinol was the only plant lignan showing seasonal variation with highest concentrations in the outdoor periods. This is in line with other findings in Norway and England (Adler et al., 2015; Nørskov et al., 2019). However, they also reported higher secoisolariciresinol and lariciresinol concentrations during the outdoor periods. Interestingly, the two mammalian lignans, enterolactone and enterodiol did not express similar trends in the present study. The concentration of enterodiol was affected by period ($P < 0.01$) as well as the interaction ($P < 0.05$). It showed a very clear trend of the concentration in biodynamic milk being highest in the outdoor periods compared with the indoor periods. Additionally, the concentration in organic milk expressed a partly similar trend, though not as pronounced as the biodynamic. Oppositely, the enterolactone concentrations were not affected by period nor the interaction effect of management and period, which is reflected in both the sum of mammalian lignans and the sum of lignans as enterolactone is found in much higher concentrations than enterodiol. This is in contrast with previous findings (Adler et al., 2015; Njåstad et al., 2014; Nørskov et al., 2019). In line with the present study, Antignac et al. (2004) also did not find any differences between the concentration of enterolactone in conventional and organic milk, but they did not include enterodiol. The differences in enterolactone and enterodiol trends in the present study is interesting from the perspective of metabolism and conversion, as the plant precursor, matairesinol is directly converted into the mammalian end product entrolactone, while secoisolariciresinol is degraded into enterodiol first (Höjer et al., 2012; Peterson et al., 2010). It has been argued that high concentrations of mammalian lignans are related to the white clover content (Andersen et al., 2009a; Höjer et al., 2012; Steinshamn et al., 2008). Hence, the present study's higher concentration of enterodiol during the outdoor periods, is most likely explained by the higher concentration as well as bioavailability of white clover in the fresh grass, which is expected to constitute a major part of the feed during the outdoor period in organic and especially biodynamic farming.

The biodynamic milk in the present study is collected from the same dairy but from two different farms. All pastures on these farms are characterised by clover grassland with a lot of different naturally occurring herbs and flowers grown biodynamically to achieve great biodiversity. The accessibility to the high amounts of clover in the clover grasslands is most likely partly responsible for the high levels of phytoestrogens (especially isoflavones) found in biodynamic milk. Additionally, Adler et al. (2015) argues that the origin of the equol content in milk from different management systems is closely related to the feed and the proportion of concentrate in the feed, which, beside the differences in concentrations, is also of great interest. They argue that the equol in organic (and in the case of present study also biodynamic) milk originates from the forage, whereas the equol content in conventional milk is mainly from the soybeans in the concentrate (Adler et al., 2015; Nørskov et al., 2019).

Additionally, the protein content was significantly higher in the biodynamic milk compared with both conventional and organic, suggesting slightly more concentrated samples, which would probably also be reflected in a lower general milk yield which is often a characteristic of biodynamic milk (Baars et al., 2019). This may also influence the absolute phytoestrogen concentrations but not the relative composition. Three of the six organic milk lines included in present study are categorised as grass milks as they are from cows exclusively fed with grass, grass field products and herbs from the pastures (Table 1). The differences in the feeding strategies within the group of organic samples may be one of the reasons for its observed relatively large variation in the determined concentrations of the different phytoestrogens. By comparing the organic grass milk lines as an individual group to the other management system, we found phytoestrogen concentrations to be slightly higher here than the levels observed for the organic mixed feed lines, though lower than the biodynamic (data not shown). However, concentrations of the mammalian lignans were higher in organic grass compared with levels for milk lines from all other management systems. Especially, the concentration of enterolactone was almost twice as high in milk from organic grass milk lines as compared with milk from other management systems, suggesting that the botanical composition of the grass fed to the organic cows differentiate from the other management systems. White clover has been shown to be related to a higher content of lignans compared with red clover, which potentially may be a part of the explanation of these trends (Steinshamn et al., 2008). Adler et al. (2015) also argues, that white clover is more often used in pastures, while red clover is typically used for grasslands intended for harvesting. Hence, it would be very interesting to study this in more detail, as it indicates that feeding strategies possess an important role in the phytoestrogen composition in milk.

For the presented results, it needs to be noted that they are based on a relatively limited number of samples from each represented milk line, and hence the statistical models were not very strong. As a consequence, the data are also rather unbalanced regarding both the grouping into management systems and seasonal periods. Especially, the seasonal aspect with periodic grouping was based on very few samples within each combination of period and management system, which may affect the outcome of the analyses. Alternatively, the milk lines could be analysed and compared individually instead of the grouping into management system, but this would require more samples.

5. Conclusion

This study revealed significant differences in phytoestrogen concentrations and their composition across the farm management systems, conventional, organic and biodynamic, as well as seasonal periods. The concentration of the total isoflavones was observed to be highest in biodynamic dairy lines. The levels of plant isoflavones were here, however, not significantly different between conventional and organic milk, which contradicts the findings in other studies, though both daidzein and genistein were significantly higher in biodynamic milk. This may be caused by differences in feed composition and access to pastures with varying botanical compositions.

Additionally, the present study was carried out on dairy milk lines, and therefore some of earlier observed differences reported on, e.g., individual cow's milk may not manifest into the broader dairy milk line perspective. The concentrations of the mammalian lignan, equol, was approximately 2-fold higher in the biodynamic dairy milk lines than organic, which again was approximately 2-fold the level observed in the conventional milk lines. Biodynamic milk lines were more affected by seasonal periods than both organic and conventional, with higher equol content during the indoor period and higher lignan content during outdoor period. The higher phytoestrogen concentrations in biodynamic milk is most likely a consequence of the distinct botanical composition in the used pasture as well as possibly higher access to clover and legumes, also in comparison with organic production.

Credit author statement

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Nina Aagaard Poulsen: conceptualization, methodology, formal analysis, resources, writing original draft, supervision, project administration.

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Declaration of competing interest

None.

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