Modification of the sterol profile in milk through feeding

Modification of the sterol profile in milk through feeding. Duong, et al. In this study we investigated whether feeding cattle different diets can influence the resulting phytosterol profile in the milk produced. Five experiments were conducted using common cattle feeds as well as selected formulated feeds and although some statistically significant changes were observed for some individual phytosterols in milk under the different feeding systems, the levels found in the milk were insignificant compared to daily intake recommendations of phytosterols for humans. However, one feeding experiment using a rumen protected feed with high phytosterol levels resulted in a decreased transfer of cholesterol to the milk by as much as 20% although further work is required to confirm these preliminary results.

Modification of the sterol profile in milk through feeding

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ABSTRACT

The fortification of milk with phytosterols is an increasingly common practice to enhance the sterol profile and offer consumers potential health benefits. This study investigated whether cattle feed can influence the profile of phytosterols and cholesterol in the milk produced as an alternative to direct fortification of milk. Five experiments were performed using feeds commonly used by Australian dairy farmers and selected formulated protected feeds. Statistical significances were observed for some individual plant sterols and cholesterol in milk under these differing feeding regimes compared to the respective controls. In the case of the phytosterols where the daily recommended consumption is typically 2 g per day, the total phytosterols were <0.12 mg/100 mL of milk. An experiment using a rumen protected feed with high phytosterol levels suggested a decreased transfer of cholesterol to the milk by as much as 20% although further work is required to confirm these preliminary results. Overall, the study suggests that different feeding practices have minimal impact on the resulting sterol profile of the milk.

Keywords: phytosterols, milk fortification, rumen protected feed, cholesterol

INTRODUCTION

Over recent decades, health conscious consumers have influenced the dairy market to supply a broad variety of milk products. This is generally achieved by the removal or addition of various compounds including fats, vitamins and omega fatty acids (**FAs**) (Ben-Ishay et al., 2017; Yeh et al., 2017). More recently, there has been an increased interest in the consumption of phytosterols which are produced by plants and have a chemical structure similar to cholesterol (Gylling et al., 2014). In humans, the consumption of high levels of phytosterols reduces the uptake of dietary cholesterol and to compensate, the body produces cholesterol for metabolic functions (Gylling et al., 2014; Phang and Garg, 2014; Moreau et al., 2018). Detection of the cholesterol precursor lanosterol in the bloodstream is generally an indicator of

metabolic or dietary blood cholesterol (Gylling et al., 2014). Phytosterols have also been associated with phytoestrogens due to similarities in their chemical structures. However, the classification of phytosterols as part of the phytoestrogen group is potentially controversial since some *in vitro* experiments (Mellanen et al., 1996; Waalkens-Berendsen et al., 1999) have demonstrated that phytosterols do not appear to activate estrogen receptors that would otherwise influence fertility (Rideout et al., 2012).

Fortification of milk nutrients is usually achieved by the direct addition of the nutrients to the final product with this technique recently shown to enhance vitamin E, selenium and levels of different FAs in milk (Leduc et al., 2017; Meignan et al., 2017; Pfrimer et al., 2018). An alternative to the direct addition of nutrients can be achieved *via* natural means such as modification of the diet of the cows or biofortification which can reduce the possibility of over-fortifying milk thus reducing the risk of potential health problems from over-consumption of the added macronutrients. This is particularly important since some studies have shown that a high phytosterol diet can reduce the levels of beneficial fat soluble vitamins in blood plasma in humans (Goncalves et al., 2011; Gylling et al., 2014).

Enhancement of the FA profile in milk through biofortification has enabled an understanding of the influence on milk quality, with a balanced nutritional diet the key to successful enhancement (Kalač and Samková, 2010). Given that phytosterol FA esters (i.e. sterols with a FA moiety) comprise the major sterol conjugates found in the lipid portion of many plants (Moreau et al., 2018), feeding studies related to the modification of the FA profile of milk may offer some insight into the possibility of phytosterol biofortification through feeding and the challenges therein. Several researchers have documented the impact of various feed types on the resulting influence on the FA profiles of milk. These experiments have shown that cattle breed, FA content in the feed and ruminal digestion are all factors that can influence the FA content in milk (Egger et al., 2009; Hristov et al., 2011; Baldinger et al., 2013; Samková et al., 2014). For example, Steinshamn (2010) reviewed experimental data from feeding trials

using a variety of grassland legumes such as white, red clover and lucerne and demonstrated that in general, a difference in feed resulted in changes to the FA composition of milk when compared to the control.

Rumen protected feeds are formulated to be protected from degradation in the rumen allowing the cattle to adsorb the intended nutrients further down the digestive tract. Protection can be achieved by various methods that are primarily based on encapsulating the feed in order to increase the rumen uptake of oilseeds and oilseed meals and rumen protection has been studied and used with varying degrees of success for over 30 years. Studies have shown that milk quality and the FA composition of beef fat can be influenced by using rumen protected feeds containing a broad range of macro-nutrients, FAs and amino acids (Gulati et al., 2000; Wang et al., 2013; Gómez et al., 2015; Pineda and Cardoso, 2015).

There are a number of experiments that have reported on the concentrations of phytosterols in humans and how these are influenced by diet (Rideout et al., 2012; Berciano and Ordová S, 2014; Huang et al., 2017; Moreau et al., 2018). However, there are few recent reports in the scientific literature that have documented the phytosterol content in ruminant milk (Jensen, 2002). Furthermore, there are no published studies reporting how feeding practices might influence the phytosterol content of ruminant milk. The aim of the present study was therefore to determine the potential to enhance the sterol content in milk. This was achieved through five controlled feeding experiments in which the cows were subjected to a variety of different diets.

MATERIALS AND METHODS

Feeding Experiments

Four feeding experiments were conducted by researchers from the Department of Economic Development, Jobs, Transport and Resources (formerly known as the Department of

Primary Industries), at the Ellinbank facilities in Victoria, Australia. This included: (1 and 2) two pasture supplementation feeding experiments (Auldist et al 2013; Akbaridoust et al. 2014; Auldist et al., 2014); (3) a tannin and cotton seed oil feeding experiment (Aprianita et al., 2014); (4) a grape marc feeding experiment (Moate et al., 2014); and (5) a rumen protected feeding experiment that was conducted at Naturale Pty Ltd in Ringarooma, Tasmania in 2015. A more detailed description of each of the feeding experiments is presented in the supplementary material including tables of the feed types and feed amounts.

Pasture Supplementation Experiment I. In this experiment, which was performed in April/May 2010, three main feeding treatments were investigated to assess differences in the feeding mode and the type of feed supplements. At the start of this experiment, the cows were 227 ± 72.8 (mean ± standard deviation) days in milk (**DIM**). In this experiment, the feeding regimes included a standard practice control (**SPC**) treatment (72 cows), and two pasture supplementation experiment (**PSE**) treatments (72 cows each) where cattle in all groups were initially grazed on ryegrass pasture where the pasture allowance per cow was approximately 14 kg dry matter (**DM**) per day. In the SPC group, the feed was supplemented twice daily with barley grain fed in the dairy and pasture silage fed in the paddock. Similar to the SPC group, cows in the PSEIa treatment were also supplemented with barley grain and pasture silage, but these components were fed as a mixed ration on a feed pad. For the third treatment, PSEIb, cows were offered a supplement of barley grain, maize, lucerne hay and maize silage which was also offered as a mixed ration on a feed pad. To evaluate the influence of feeding amount, the groups were further divided into four groups of nine cows which were each fed 6, 8, 10, or 12 kg of supplement based on the DM content per day.

Pasture Supplementation Experiment II. This feeding experiment was performed in October/November 2010 to assess differences in the feeding mode and the type of feed supplements. At the start of this experiment, the cows were 70 ± 15.2 (mean ± standard deviation) DIM. Three main feeding treatments were investigated including a SPC treatment

where cattle were fed on ryegrass pasture supplemented with wheat grain in the dairy and pasture silage in the paddock (64 cows). A second feeding treatment based on pasture supplementation (PSEIIa) involved cattle fed on the same ryegrass pasture as the SPC treatment, with the cow's diet supplemented with wheat and corn grain, corn silage and lucerne hay fed as a mixed ration on a feed pad (64 cows). A third dietary treatment (PSEIIb) was investigated that involved cattle grazing ryegrass pasture diet supplemented with the same mixed ration as PSEIIa but in which some of the wheat had been replaced with solvent-extracted canola meal (32 cows). The pasture allowance for all treatments was approximately 14 kg DM/day for each cow. To evaluate the influence of feeding amount, the SPC and PSEIIa groups were divided into four groups of eight cows (per replicate) which were each fed 8, 10, 12 or 14 kg of supplement based on the DM content per day. Cows on the PSEIIb treatment were divided into two groups eight cows (per replicate) and were each fed 12 and 14 kg of supplement based on the DM content per day.

Tannin and Cotton Seed Oil Feeding Experiment. This experiment was conducted in September 2010 and at the start of this experiment, the cows were 39 ± 13 (mean ± standard deviation) DIM. Cows were fed a control diet, or the control diet supplemented with either 800 g/day of tannin (**TANN**) from black wattle, 800 g/d of cotton seed oil (**CSO**) or with 400 g/day each of tannin and cotton seed oil (**TCSO**). The experimental design was performed using a Latin square scheme for a duration of 16 weeks. Each diet was implemented to the cows for four weeks before moving onto the next respective diet.

Grape Marc Feeding Experiment. This experiment was conducted during March 2011 and at the start of this experiment, the cows were 203 ± 72.8 (mean ± standard deviation) DIM. In this experiment there were three dietary treatments: control, dried grape marc (**DGM**) and wet grape marc (**WGM**) feeds. All cows in the study were placed on the control diet for the first three weeks of the experiment before they were moved to their respective intended diets. The daily control diet consisted of 14 kg DM of alfalfa hay and 4.3 kg DM of concentrate mix,

the DGM diet consisted of 9 kg DM of alfalfa hay, 4.3 kg DM of concentrate mix and 5 kg DM of DGM, and the WGM diet consisted of 9 kg DM of alfalfa hay, 4.3 kg DM of concentrate mix and 5 kg DM of WGM.

Rumen Protected Feeding Experiment. This experiment was conducted in mid-September 2015 for a period of four weeks, the cows were 195 ± 30 (mean ± standard deviation) DIM. In this experiment, seven cows were used with four dietary feeding treatments including control, sterol protected (**SP**) feed, omega protected (**OP**) feed containing eicosapentaenoic acid (**EPA**) and docosahexaenoic acid (**DHA**) and canola/soybean protected (**CSP**) feed. Both the sterol and soybean/canola protected feeds contained high levels of plant sterols with the sterol protected feed consisting of a phytosterol fatty ester paste while the soybean/canola consisting of natural phytosterols originating from the components. All the animals were placed on the control diet on the first two weeks before moving to their respected diets. The daily control diet consisted of 3.6 kg DM of dairy herd concentrate (60% corn and 40% wheat), 3.5 kg DM of maize silage and approximately 10 kg DM of pasture. The daily protected feeds were the same as the control diet with the addition of 620 g of protected omega feed, 800 g of protected sterol feed, or 1000 g of protected soybean/canola feed. Rumen protection of the feed was achieved using a protein encapsulation process with cinnamaldehyde (a non-toxic food additive) *via* a process similar to that reported by Gulati et al. (1999). In brief, the sterol fatty esters, canola oil/soy bean oil or omega EPA and DHA were emulsified before cinnamaldehyde was added to the emulsified lipids to create cross-linkages between the emulsified oil and proteins. The mixture was then dried until it was a free-flowing powder. This type of protection allows the feed to avoid dehydrogenation in the rumen during the digestion process of the cattle (Gulati et al., 2000a).

In each set of experiments, representative samples of morning and afternoon milk were collected from individual cows over the last two days of the experiment using an inline meter (DeLaval International, Tumba, Sweden). Milk samples were combined and stored in opaque

plastic containers and were immediately frozen at -20°C prior to analysis to reduce minimal loss of sterol content. The authors acknowledge due to the length of storage there is an expectant change to the milk fat or composition (Chang et al., 2012; García-Lara et al., 2012). However, since all the samples within the experiments were collected, stored and analyzed at the same time, any degradation of the milk within the experiments would be similar and therefore comparisons are deemed to be valid. In addition, duplicate samples and comparisons to literature reports were found to be comparable for both the cattle feed and milk with regards to sterol content (Gorban and Izzeldin, 1999; Piironen et al., 2002a; Reklewska et al., 2002; Ruibal-Mendieta et al., 2004; Foods standards Australia New Zealand, 2010).

Reference Standards and Reagents

Reference standards with a purity assay greater than 95% were purchased from Sigma Aldrich (Sydney Australia) including: cholesterol, stigmasterol, stigmastanol, brassicasterol, lathosterol, lanosterol, 5b-cholestan-3a-ol, β-sitosterol, and campesterol (purity assay 65%). All reference standards were prepared in heptane as 500 mg/L stock solutions. An acid hydrolysis solution of 8 M hydrochloric acid diluted in ethanol was prepared prior to analysis. A saponification solution of 5 M potassium hydroxide (Sigma Aldrich, Sydney), with the potassium hydroxide dissolved in water and made up in ethanol to volume. N-O-bis-(trimethylsilyl) trifluoroacetamide (**BSTFA**) with 1% trimethylchlorosilane was obtained from Grace Davison; n-heptane, pyridine and cyclohexane were obtained from Merck (Melbourne, Australia); and boiling chips (BDH, Sydney, Australia). Ultrapure, Type 1 water was used throughout the experiments and was obtained using a Millipore water purification system (Element A10).

Extraction of Phytosterols from Milk

The milk and cattle feed were analyzed for their phytosterol content as per the method described by Duong et al., (2018). In brief: 5 mL of thawed milk was spiked with a surrogate

standard (5β-cholestan-3α-ol), followed by the addition of 5 mL heptane which was then hydrolyzed using hydrochloric acid (4 mL of 8 M). The solution was incubated in a water bath at 80˚C for 30 minutes and then allowed to cool to room temperature prior to the addition of 20 mL ethanolic potassium hydroxide (5 M). The mixture was again incubated for 30 minutes and allowed to cool to room temperature prior to the addition of 4 mL of Ultrapure, Type 1 water. The mixture was vortexed and allowed to settle or until two liquid layers were obtained. The organic layer was collected and evaporated to 1 mL using nitrogen gas and transferred to a sample vial. The extract in the sample vial was once again evaporated to dryness using nitrogen gas and derivatized using 300 µL of BSTFA and 700 µL of a toluene/pyridine mixture before the vial was crimped, mixed and incubated at 80˚C for 20 minutes prior to analysis.

GC-MS/FID Analysis

An Agilent 7890 gas chromatograph (**GC**) coupled with a 5975c mass spectrometry (**MS**) detector and a flame ionization detector (**FID**), using a HP-5MS capillary column (5%-phenyl-methylpolysiloxane 30 m × 0.25 µm × 0.25 µm film thickness) were used to perform the analyses. The following oven program was used: initial oven temperature 245ºC held for 0.5 minutes; followed by an increase to 265ºC at 2ºC/min then to 290ºC at 3.5 ºC/min held for 8 minutes; a 7.5-minute post-run program at 240ºC with a back-flush flow at 24.6 psi was then applied for a total run time of 32 minutes. Sample injection was performed at 310ºC for all samples with injection volumes of: 1 µL with a 1:20 split for cholesterol analysis in milk; 2 µL split-less for phytosterol analyses in milk; and 1 µL with a 1:5 split for animal feed samples.

Statistical Analysis

A one-way analysis of variance (**ANOVA**) was applied to the data which included comparisons of the pooled sterol contents between the feeding type and the pooled sterol contents from the feeding rates where applicable. This comparison allowed for the identification of any significant differences within the group that could demonstrate the effect of feed upon milk with regards to sterol content. In addition, where significant differences were found in any

of the analyses, a post-hoc (t-test) was performed to determine and identify the possible significance. Statistical calculations were performed using IBM SPSS statistics version 23 software and statistically significant differences between treatments were declared when $p < 0.05$.

RESULTS AND DISCUSSION

Pasture Supplementation Experiment I

A summary of the detected sterol levels measured in the milk from the first mixed ration feeding experiment is presented in Table 1 with the results a combined mean from the different feeding rates for the same treatment. In this experiment, β-sitosterol, brassicasterol campestanol and stigmasterol were detected at <0.02 mg/100 mL with total phytosterols detected at <0.12 mg/100 mL. There were differences ($P < 0.05$) in the lathosterol levels for PSEIa and PSEIb with mean lathosterol levels in milk produced under the PSEIa regime 15% higher than in milk produced under the PSEIb regime. Lathosterol was not detected in the feed samples and, in this case, both the SPC and PSEIa shared the same feeding regime with the only difference in the method of offering the supplements to the cows. This change was not observed for the SPC where the cows on this diet were fed barley grain (75%) and ryegrass silage (25%) supplements in the paddock whereas those on the PSEIa were given the same feed but from concrete feed pads. The results suggest that levels of lathosterol in milk may be influenced by the difference in the feed from PSEIa and PSEIb even though lathosterol was not present in either feed. The main difference in the feed composition is the presence of maize grain and maize silage in the PSEIb which may have contributed to the lower the lathosterol content in the milk although the mechanism for this is unclear.

>>>Insert Table 1

The effects of the quantity of feed given to the animals was explored with the results calculated as the mean value of all the same feed rates over the different feeding regimes. As

shown in Table 2, there were no effects on the sterol contents in milk as a result of different feeding rates. In a concurrent study, milk yield results were shown to significantly increase at higher rates (Auldist et al., 2013) so the results in the present study suggest that an increase in milk yield or the amount of feed does not appear to influence total phytosterol content with the exception of lathosterol content.

>>>Insert Table 2

Pasture Supplementation Experiment II

A summary of the results from the second mixed ration feeding experiment are presented in Table 3, which shows a comparison between the mean sterol content of each feeding regime and feeding rate. Similar to the results in the first mixed ration feeding experiment, β-sitosterol, brassicasterol campestanol and stigmasterol were detected at <0.02 mg/100 mL with total phytosterols detected at <0.12 mg/100 mL. A comparison between the three feeding regimes of this experiment showed differences for cholesterol, lathosterol, campesterol and lanosterol. A further post-hoc analysis was performed on these individual sterols and the results indicated that the levels in milk were influenced by the differences in feeding regimes. In milk produced under the PSEIIa diet, cholesterol and lanosterol contents were lower when compared to milk produced under the PSEIIb diet. The main difference between the PSEIIa and PSEIIb is the addition of protein in the form of canola meal (16%) and a lower amount of crushed wheat in the PSEIIb diet (reduced from 39% to 23%). Although the reason for the increased cholesterol and lanosterol in the milk produced by cows fed the higher protein diet are unclear, there is some evidence that lanosterol can reverse protein aggregation (Zhao et al., 2015). The results also show that milk produced by cows fed under both PSEIIa and PSEIIb contained the same mean lathosterol content which was lower than the milk from the SPC diet. Moreover, the campesterol level in milk was observed to be highest in the SPC group.

A comparison of total phytosterol content in the feed at the 13.5 kg DM rate was 13200, 12300 and 13500 mg/kg for the control, PSEIIa and PSEIIb respectively with trace levels of

cholesterol (Duong et al., 2018). This experiment suggests that the addition of protein into the feed in the form of solvent extracted canola meal may increase the levels of cholesterol and lanosterol in the milk produced when compared to the PSEIIa milk. Although the reasons for this are unclear, research by Strzałkowska et al., (2010) showed that in a year-long experiment under the same feeding regime, cholesterol content was influenced by the time of year, the stage of lactation, and somatic cell count.

>>>Insert Table 3

The effects of the quantity of feed given to the cows was also explored with the results calculated as the mean value of all the same feed amounts over the different feeding regimes. In this case, statistical comparisons between the mean feed rates across the different regimes showed differences in the levels of campesterol as shown in Table 4. However, the results overall were considered to be insignificant between the groups.

>>>Insert Table 4

Tannin and Cotton Seed Oil Feeding Experiment

In the tannin and cotton seed oil feeding experiment, there were no differences in the sterol profiles in the milk produced by the cows given the TANN, CSO or TCSO feeds as shown in Table 5. Even though CSO is a naturally rich source of phytosterols, its addition to the cattle diet did not enhance total phytosterol content in the milk in this study. In humans, the consumption of high levels of phytosterols is usually reflected in the plasma levels and a decrease in LDL cholesterol is also observed (Ostlund et al., 1999; Kritchevsky and Chen, 2005). It is suspected that the same affect may result when cows are fed a diet supplemented in phytosterols that is in excess of their normal consumption. Future work to analyse the blood plasma may aid in the understanding of the cows' metabolism of phytosterols.

>>>Insert Table 5

Grape Marc Feeding Experiment

The grape marc feeding experiment showed significant differences in the lanosterol levels between the feeding regimes as shown in Table 6. It was observed that the mean lanosterol content was highest in the milk from the animals fed on the control diet and lowest in the milk from those feds on the DGM feed, however, this was not reflected in the cholesterol results for the different feed types. Examination of the phytosterol content in the respective diets showed that the control diet contained the lowest amount of phytosterols with 4254 mg/kg compared to the WGM and DGM which contained 9922 mg/kg (Duong et al., 2018). The results suggest that high phytosterol levels may affect the endogenous synthesis of lanosterol as this is generally observed in human subjects ingesting a high plant sterol diet (Ostlund, 2002; Kritchevsky and Chen, 2005).

In this case, the WGM and DGM diets were fed at the same weight based on the amount of DM in the feed the lanosterol. This further suggests that the moisture level in the feed may have some influence on the metabolism and digestion of the feed, that may decrease phytosterol absorption and increase endogenous lanosterol levels in the milk which are a metabolic precursor for cholesterol (Jäpelt and Jakobsen, 2013). The total amount of phytosterols detected were less than 0.12 mg/100 mL in all cases, which suggests that the feeding of either form of grape marc to dairy cows did not enhance total phytosterol levels in milk.

>>>Insert Table 6

Rumen Protected Feeding Experiment

Similar to the previous experiments, the total phytosterol content found in all milk samples under the rumen protected feeds was <0.12 mg/100 mL. As shown in Table 7, significantly higher levels of cholesterol, lanosterol and lathosterol levels in the milk were found in animals fed the OP diet with milk produced from the SP and CSP feed supplements resulting in the lowest cholesterol content. In humans, the consumption of phytosterols is suggested to reduce dietary cholesterol and regulate metabolic synthesis of cholesterol. The present study suggests that similar to humans, the consumption of high sterol contents by cattle may influence the

metabolic synthesis of cholesterol resulting in a reduced cholesterol content expressed in the bovine milk (Lichtenstein and Deckelbaum, 2001; Kritchevsky and Chen, 2005; Ostlund, 2007). Given that the lathosterol levels in the milk from the SP and the CSP feed experiments were both lower than the control and OP feed, this is in accordance with observations in humans where low levels of lathosterol in blood plasma are consistent with lower cholesterol.

The phytosterol content in the protected portion of the OP, CSP and SP feeds contained total plant sterols of 1319, 2593 and 6673 mg per day respectively. The results show that levels of cholesterol in the milk produced by cows fed the high plant sterol SP and CSP diets were 22% and 11% lower than the control respectively. In the case of the OP feed, which contained relatively high amounts of protected phytosterol, it also contained 799 mg of cholesterol per day which may have interfered with plant sterol absorption.

In addition, the results for both the phytosterol and cholesterol content for the milk produced were within normal range compared to nutritional panels and previous studies (Gorban and Izzeldin, 1999; Piironen et al., 2002b; Reklewska et al., 2002; Foods Standards Australia New Zealand, 2010). Although this experiment was limited in the number of samples, particularly with regard to the OP treatment, the results indicate that cholesterol levels may be influenced by rumen protected feeding and further experiments would be needed to confirm the results. In all cases, β-sitosterol was detected at <0.02 mg/100 mL for this experiment.

>>>Insert Table 7

Overview

Overall the levels of cholesterol measured in the milk samples from five feeding experiments ranged from 10.3 to 24 mg/100 mL and the majority of the milk samples contained less than 0.12 mg/100 mL of total phytosterols. The major sterols found in the milk were cholesterol, lathosterol and lanosterol, the latter being a precursor sterol for cholesterol (Jäpelt and Jakobsen, 2013). Other plant sterols detected in the milk samples included campesterol and β-sitosterol, but at minor or trace levels. In a recent study, the phytosterol content in the cattle

feed used for this present study, lanosterol and lathosterol were not detected (Duong et al., 2018). Given that both lanosterol and lathosterol were found in the milk, it suggested that their presence in milk was a result of endogenous synthesis. In general, the rumen protected feeds containing high phytosterol contents produced milk with cholesterol levels 11-22% lower than the control.

The results of these feeding experiments demonstrate that certain feeds consumed by the cattle can influence individual sterol contents in bovine milk including lanosterol, lathosterol, campesterol and cholesterol, but only to a minor extent. Changes were observed between control groups and diets containing maize silage, maize grain, canola meal, DGM, and high sterol rumen protected feeds. The mode of feed offering was also shown to influence the levels of these sterols. However, given that the safe and beneficial recommended levels of phytosterol consumption by many food authorities is approximately 2000 mg/day (Lichtenstein and Deckelbaum, 2001; Kritchevsky and Chen, 2005), the levels determined in this study were less than 0.12 mg/100 mL which is much lower than some fortified levels of 300 mg/100 mL in milk which would require the consumption of 600 mL of milk to reach the target level (Pollak, 1953; Gerson et al., 1961; Miettinen et al., 1995; Carr et al., 2010; Truswell, 2010).

Of all the feeding experiments, the formulated SP and CSP feeding types resulted in the production of milk with a reduced cholesterol content. However, the phytosterol content in the milk produced under any of the formulated feeding programs was unchanged. In addition, the results also indicate that the phytosterol transfer from feed to the milk was not direct for the rumen protected feed unlike the transfer of omega-3 fatty acid to milk that has previously been reported (Ashes et al., 1992; Gulati et al., 1997).

Overall, our results demonstrate that the feeding of diets containing high amounts of phytosterols has an insignificant impact upon the phytosterol content of milk. Thus, the feeding of phytosterol rich feeds to cattle in order to enhance the phytosterol concentrations in milk cannot be recommended. In addition, dairy industries worldwide are generally highly regulated

with milk carefully homogenized, pasteurized and fortified to maintain consistent quality control. Thus, any natural fortification achieved on one farm will most likely be diluted during the post-farm processing of milk. These expectations were based on the results of previous studies that reported changes in the fatty acid profile as a result of feeding studies, and given that phytosterol fatty acid esters are a common sterol conjugate found in cattle feed (Dutta, 2004; Hristov et al., 2011; Samková et al., 2014).

CONCLUSIONS

The results of this research indicated total phytosterol content in milk cannot be enhanced through feed trials conducted in the experiments. Although some minor changes were observed with the levels of some individual sterols, overall natural fortification of phytosterols in milk was not achieved through these feeding trials. However, some changes were observed with respect to the cholesterol levels for the formulated sterol protected and canola/soybean oil sterol protected feed with 22% and 11% reductions respectively when compared to the control diet.

REFERENCES

Akbaridoust, G., T. Plozza, V.C. Trenerry, W.J. Wales, M.J. Auldist, F.R. Dunshea, and S. Ajlouni. 2014. Influence of different systems for feeding supplements to grazing dairy cows on milk fatty acid composition. J. Dairy Res. 81:156–163. doi:10.1017/S002202991400003X.

Aprianita, A., O.N. Donkor, P.J. Moate, S.R.O. Williams, M.J. Auldist, J.S. Greenwood, M.C. Hannah, W.J. Wales, and T. Vasiljevic. 2014. Effects of dietary cottonseed oil and tannin supplements on protein and fatty acid composition of bovine milk. J. Dairy Res. 81:183–192. doi:10.1017/S0022029914000065.

Ashes, J.R., P. St. Vincent Welch, S.K. Gulati, T.W. Scott, G.H. Brown, and S. Blakeley. 1992. Manipulation of the Fatty Acid Composition of Milk by Feeding Protected Canola Seeds. J. Dairy Sci. 75:1090–1096. doi:10.3168/jds.S0022-0302(92)77853-9.

Auldist, M.J., L.C. Marett, J.S. Greenwood, M. Hannah, J.L. Jacobs, and W.J. Wales. 2013. Effects of different strategies for feeding supplements on milk production responses in cows grazing a restricted pasture allowance. J. Dairy Sci. 96:1218–1231. doi:10.3168/jds.2012-6079.

Auldist, M.J., L.C. Marett, J.S. Greenwood, M.M. Wright, M. Hannah, J.L. Jacobs, and W.J. Wales. 2014. Replacing wheat with canola meal in a partial mixed ration increases the milk production of cows grazing at a restricted pasture allowance in spring. Anim. Prod. Sci. 54:869–878. doi:10.1071/AN13154.

Baldinger, L., W. Zollitsch, and W.F. Knaus. 2014. Maize silage and Italian ryegrass silage as high-energy forages in organic dairy cow diets: Differences in feed intake, milk yield and quality, and nitrogen efficiency. Renew. Agric. Food Syst. 29:378–387. doi:10.1017/S1742170513000252.

Ben-Ishay, N., H. Oknin, D. Steinberg, Z. Berkovich, R. Reifen, and M. Shemesh. 2017. Enrichment of milk with magnesium provides healthier and safer dairy products. npj Biofilms Microbiomes 3:24. doi:10.1038/s41522-017-0032-3.

Berciano, S., and J.M. Ordová S. 2014. Nutrition and Cardiovascular Health.

Carr, T.P., M.M. Ash, and A.W. Brown. 2010. Cholesterol-lowering phytosterols: factors affecting their use and efficacy. Nutr. Diet. Suppl. 2:59–72. doi:10.2147/NDS.S10974.

Chang, Y.C., C.H. Chen, and M.C. Lin. 2012. The macronutrients in human milk change after storage in various containers. Pediatr. Neonatol. 53:205–209. doi:10.1016/j.pedneo.2012.04.009.

Duong, S., N. Strobel, S. Buddhadasa, K. Stockham, M.J. Auldist, W.J. Wales, P.J. Moate, J.D. Orbell, and M.J. Cran. 2018. Influence of acid hydrolysis, saponification and sample

clean-up on the measurement of phytosterols in dairy cattle feed using GC-MS and GC with flame ionization detection. J. Sep. Sci. 1–10. doi:10.1002/jssc.201800484.

Dutta, P.C. 2004. Phytosterols as Functional Food Components and Nutraceuticals. CRC Press.

Foods standards Australia New Zealand. 2010. NUTTAB. Accessed.

Foods Standards Australia New Zealand. 2010. NUTTAB 2010 Electronic Database Files. Accessed October 19, 2018. http://www.foodstandards.gov.au/science/monitoringnutrients/nutrientables/nuttab/Pages/NUTTAB-2010-electronic-database-files.aspx.

García-Lara, N.R., D. Escuder-Vieco, O. García-Algar, J. De la Cruz, D. Lora, and C. Pallás-Alonso. 2012. Effect of Freezing Time on Macronutrients and Energy Content of Breastmilk. Breastfeed. Med. 7:295–301. doi:10.1089/bfm.2011.0079.

Gerson, T., F.B. Shorland, and Y. Adams. 1961. The effects of corn oil on the amounts of cholesterol and the excretion of sterol in the rat. Biochem. J. 81:584–91.

Gómez, I., J.A. Mendizabal, M. V Sarriés, K. Insausti, P. Albertí, C. Realini, M. Pérez-Juan, M.A. Oliver, A. Purroy, and M.J. Beriain. 2015. Fatty acid composition of young Holstein bulls fed whole linseed and rumen-protected conjugated linoleic acid enriched diets. Livest. Sci. 180:106–112. doi:10.1016/j.livsci.2015.07.023.

Goncalves, A., B. Gleize, R. Bott, M. Nowicki, M.J. Amiot, D. Lairon, P. Borel, and E. Reboul. 2011. Phytosterols can impair vitamin D intestinal absorption in vitro and in mice. Mol. Nutr. Food Res. 55:S303–S311. doi:10.1002/mnfr.201100055.

Gorban, A.M.S., and O.M. Izzeldin. 1999. Study on cholesteryl ester fatty acids in camel and cow milk lipid. Int. J. Food Sci. Technol. 34:229–234. doi:10.1046/j.1365-2621.1999.00254.x.

Gulati, S.. K., J.R. Ashes, and T.. W. Scott. 1999. Hydrogenation of eicosapentaenoic and docosahexaenoic acids and their incorporation into milk fat. Anim. Feed Sci. Technol. 79:57–64. doi:10.1016/S0377-8401(99)00010-3.

Gulati, S., J. Ashes, and T. Scott. 2000a. Healthier butter fat spreads better. Feed Mix 8:20–22.

Gulati, S.K., S.M. Kitessa, J.R. Ashes, E. Fleck, E.B. Byers, Y.G. Byers, and T.W. Scott. 2000b. Protection of conjugated linoleic acids from ruminal hydrogenation and their incorporation into milk fat. Anim. Feed Sci. Technol. 86:139–148. doi:10.1016/S0377-8401(00)00170-X.

Gulati, S.K., T.W. Scott, and J.R. Ashes. 1997. In-vitro assessment of fat supplements for ruminants. Anim. Feed Sci. Technol. 64:127–132. doi:10.1016/S0377-8401(96)01063-2.

Gylling, H., J. Plat, S. Turley, H.N. Ginsberg, L. Ellegård, W. Jessup, P.J. Jones, D. Lütjohann, W. Maerz, L. Masana, G. Silbernagel, B. Staels, J. Borén, A.L. Catapano, G. De Backer, J. Deanfield, O.S. Descamps, P.T. Kovanen, G. Riccardi, L. Tokgözoglu, and M.J. Chapman. 2014. Plant sterols and plant stanols in the management of dyslipidaemia and prevention of cardiovascular disease. Atherosclerosis 232:346–360. doi:10.1016/j.atherosclerosis.2013.11.043.

Hristov, A.N., C. Domitrovich, A. Wachter, T. Cassidy, C. Lee, K.J. Shingfield, P. Kairenius, J. Davis, and J. Brown. 2011. Effect of replacing solvent-extracted canola meal with high-oil traditional canola, high-oleic acid canola, or high-erucic acid rapeseed meals on rumen fermentation, digestibility, milk production, and milk fatty acid composition in lactating dairy cows. J. Dairy Sci. 94:4057–4074. doi:10.3168/jds.2011-4283.

Huang, J., M. Xu, Y.J. Fang, M.S. Lu, Z.Z. Pan, W.Q. Huang, Y.M. Chen, and C.X. Zhang. 2017. Association between phytosterol intake and colorectal cancer risk: A case-control study. Br. J. Nutr. 117:839–850. doi:10.1017/S0007114517000617.

Jäpelt, R.B., and J. Jakobsen. 2013. Vitamin D in plants: a review of occurrence, analysis, and biosynthesis. Front. Plant Sci. 4:136. doi:10.3389/fpls.2013.00136.

Jensen, R.G. 2002. The Composition of Bovine Milk Lipids: January 1995 to December 2000. J. Dairy Sci. 85:295–350. doi:10.3168/jds.S0022-0302(02)74079-4.

Kalač, P., and E. Samková. 2010. The effects of feeding various forages on fatty acid

composition of bovine milk fat: A review. Czech J. Anim. Sci. 55:521–537.

Kritchevsky, D., and S.C. Chen. 2005. Phytosterols-health benefits and potential concerns: A review. Nutr. Res. 25:413–428. doi:10.1016/j.nutres.2005.02.003.

Leduc, M., M.-P. Létourneau-Montminy, R. Gervais, and P.Y. Chouinard. 2017. Effect of dietary flax seed and oil on milk yield, gross composition, and fatty acid profile in dairy cows: A meta-analysis and meta-regression. J. Dairy Sci. 100:8906–8927. doi:https://doi.org/10.3168/jds.2017-12637.

Lichtenstein, A.H., and R.J. Deckelbaum. 2001. Stanol/sterol ester-containing foods and blood cholesterol levels: A statement for healthcare professionals from the nutrition committee of the council on nutrition, physical activity, and metabolism of the American Heart Association. Circulation 103:1177–1179. doi:10.1161/01.CIR.103.8.1177.

Meignan, T., C. Lechartier, G. Chesneau, and N. Bareille. 2017. Effects of feeding extruded linseed on production performance and milk fatty acid profile in dairy cows: A meta-analysis. J. Dairy Sci. 100:4394–4408. doi:https://doi.org/10.3168/jds.2016-11850.

Mellanen, P., T. Petänen, J. Lehtimäki, S. Mäkelä, G. Bylund, B. Holmbom, E. Mannila, A. Oikari, and R. Santti. 1996. Wood-Derived Estrogens: Studiesin Vitrowith Breast Cancer Cell Lines andin Vivoin Trout. Toxicol. Appl. Pharmacol. 136:381–388. doi:10.1006/taap.1996.0046.

Miettinen, T.A., P. Puska, H. Gylling, H. Vanhanen, and E. Vartiainen. 1995. Reduction of serum cholesterol with sitostanol-ester margarine in a mildly hypercholesterolemic population.. N. Engl. J. Med. 333:1308–1312. doi:10.1056/NEJM199511163332002.

Moate, P.J., S.R.O. Williams, V.A. Torok, M.C. Hannah, B.E. Ribaux, M.H. Tavendale, R.J. Eckard, J.L. Jacobs, M.J. Auldist, and W.J. Wales. 2014. Grape marc reduces methane emissions when fed to dairy cows. J. Dairy Sci. 97:5073–5087. doi:10.3168/jds.2013-7588.

Moreau, R.A., L. Nyström, B.D. Whitaker, J.K. Winkler-Moser, D.J. Baer, S.K. Gebauer, and

K.B. Hicks. 2018. Phytosterols and their derivatives: Structural diversity, distribution, metabolism, analysis, and health-promoting uses. Prog. Lipid Res. 70:35–61. doi:https://doi.org/10.1016/j.plipres.2018.04.001.

Ostlund, R.E. 2002. Phytosterols in Human Nutrition. Annu. Rev. Nutr. 22:533–549. doi:10.1146/annurev.nutr.22.020702.075220.

Ostlund, R.E. 2007. Phytosterols, cholesterol absorption and healthy diets. Lipids 42:41–45. doi:10.1007/s11745-006-3001-9.

Ostlund, R.E., C.A. Spilburg, and W.F. Stenson. 1999. Sitostanol administered in lecithin micelles potently reduces cholesterol absorption in humans. Am. J. Clin. Nutr. 70:826–831.

Pfrimer, K., E. Ferriolli, P.L. Takeuchi, M.S.V. Salles, A. Saran-Netto, M.A. Zanetti, L.C. Roma-Junior, C.B.M. Braga, F.A. Domenici, Y.M.L. Valim, A.B.P. Paschoalato, L.F. Marchi, A.E.C.S. Azzolini, E.A. Donadi, E. Martinez, and H. Vannucchi. 2018. Effects of the Consumption of Milk Biofortified with Selenium, Vitamin E, and Different Fatty Acid Profile on Immune Response in the Elderly. Mol. Nutr. Food Res. 62:1–7. doi:10.1002/mnfr.201700307.

Phang, M., and M. Garg. 2014. Chapter 16 - Combined Phytosterol and Fish Oil Therapy for Lipid Lowering and Cardiovascular Health. doi:10.1016/B978-0-12-404610-8.00016-5.

Piironen, V., J. Toivo, and A.M. Lampi. 2002a. Plant sterols in cereals and cereal products. Cereal Chem. 79:148–154. doi:10.1094/CCHEM.2002.79.1.148.

Piironen, V., J. Toivo, and A.M. Lampi. 2002b. New data for cholesterol contents in meat, fish, milk, eggs and their products consumed in Finland. J. Food Compos. Anal. 15:705–713. doi:10.1006/jfca.2002.1095.

Pineda, A., and F.C. Cardoso. 2015. Effects of rumen-protected choline with calcium salts of long chain fatty acids on milk yield and milk composition of middle and late lactation Holstein cows. Livest. Sci. 175:37–46. doi:10.1016/j.livsci.2015.02.005.

Pollak, O.J. 1953. Reduction of blood cholesterol in man. Circulation 7:702–706. doi:10.1161/01.CIR.7.5.702.

Reklewska, B., A. Oprządek, Z. Reklewski, L. Panicke, B. Kuczyńska, and J. Oprządek. 2002. Alternative for modifying the fatty acid composition and decreasing the cholesterol level in the milk of cows. Pages 235–243 in Livestock Production Science. Elsevier.

Rideout, T.C., C.P.F. Marinangeli, and A.B. Awad. 2012. Regulatory approval of plant sterols in Canada: Implications for health care and clinical practice. Can. J. Diet. Pract. Res. 73:31–34. doi:10.3148/73.1.2012.31.

Ruibal-Mendieta, N.L., R. Rozenberg, D.L. Delacroix, G. Petitjean, A. Dekeyser, C. Baccelli, C. Marques, N.M. Delzenne, M. Meurens, J.L. Habib-Jiwan, and J. Quetin-Leclercq. 2004. Spelt (Triticum spelta L.) and winter wheat (Triticum aestivum L.) wholemeals have similar sterol profiles, as determined by quantitative liquid chromatography and mass spectrometry analysis. J. Agric. Food Chem. 52:4802–4807. doi:10.1021/jf049772v.

Samková, E., J. Čertíková, J. Špička, O. Hanuš, T. Pelikánová, and M. Kváč. 2014. Eighteen-carbon fatty acids in milk fat of Czech Fleckvieh and Holstein cows following feeding with fresh lucerne (Medicago sativa L.). Anim. Sci. Pap. Reports 32:209–218.

Steinshamn, H. 2010. Effect of forage legumes on feed intake, milk production and milk quality - a review. Anim. Sci. Pap. Reports 28:195–206.

Strzałkowska, N., A. Jóźwik, E. Bagnicka, J. Krzyżewski, R. G. Cooper, and J.O. Horbańczuk. 2010. Factors affecting the cholesterol content of milk of cows fed conserved feeds in a TMR system throughout the year. Mljekarstvo 60:273–279.

Truswell, A.S. 2010. Cholesterol and beyond: The Research on Diet and Coronary Heart Disease 1900-2000. Springer Science & Business Media.

Waalkens-Berendsen, D.H., A.P.M. Wolterbeek, M.V.W. Wijnands, M. Richold, and P.A. Hepburn. 1999. Safety evaluation of phytosterol esters. Part 3. Two-generation reproduction study in rats with phytosterol esters - A novel functional food. Food Chem.

Toxicol. 37:683–696. doi:10.1016/S0278-6915(99)00056-3.

Wang, D.M., C. Wang, H.Y. Liu, J.X. Liu, and J.D. Ferguson. 2013. Effects of rumen-protected γ-aminobutyric acid on feed intake, lactation performance, and antioxidative status in early lactating dairy cows. J. Dairy Sci. 96:3222–3227. doi:10.3168/jds.2012-6285.

Yeh, E.B., D.M. Barbano, and M.A. Drake. 2017. Vitamin Fortification of Fluid Milk. J. Food Sci. 82:856–864. doi:10.1111/1750-3841.13648.

Zhao, L., X.-J. Chen, J. Zhu, Y.-B. Xi, X. Yang, L.-D. Hu, H. Ouyang, S.H. Patel, X. Jin, D. Lin, F. Wu, K. Flagg, H. Cai, G. Li, G. Cao, Y. Lin, D. Chen, C. Wen, C. Chung, Y. Wang, A. Qiu, E. Yeh, W. Wang, X. Hu, S. Grob, R. Abagyan, Z. Su, H.C. Tjondro, X.-J. Zhao, H. Luo, R. Hou, J. Jefferson, P. Perry, W. Gao, I. Kozak, D. Granet, Y. Li, X. Sun, J. Wang, L. Zhang, Y. Liu, Y.-B. Yan, and K. Zhang. 2015. Lanosterol reverses protein aggregation in cataracts. Nature 523:607.

Table 1: Sterol contents from milk produced by cows fed the pasture supplementation experiment I diet: influence of feed type

Sterol[#]	Feeding regime*	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol	SPC	49	14.00	0.37	10.65	18.98
	PSEIa	49	14.44	0.35	10.41	21.21
	PSEIb	51	14.69	0.32	10.95	21.39
	Average		14.38	0.20	10.41	21.39
Lathosterol	SPC	49	0.18	<0.02	0.09	0.30
	PSEIa	49	0.20[a]	<0.02	0.08	0.30
	PSEIb	51	0.17[b]	<0.02	0.08	0.34
	Average		0.18	<0.02	0.08	0.34
Campesterol	SPC	49	0.04	<0.02	0.03	0.12
	PSEIa	49	0.04	<0.02	<0.02	0.07
	PSEIb	51	0.04	<0.02	0.02	0.10
	Average		0.04	<0.02	<0.02	0.12
Lanosterol	SPC	49	0.24	<0.02	0.13	0.55
	PSEIa	49	0.22	<0.02	0.14	0.37
	PSEIb	51	0.21	<0.02	0.02	0.41
	Average		0.22	<0.02	0.02	0.55

[#]Total phytosterols, campestanol, stigmasterol, stigmastanol and β-sitosterol were less than the limit of reporting. For each type of sterol, means followed by different superscripts were significantly within the group and identified specifically after a post-hoc analysis between the superscripted treatment ($p < 0.05$). *Pasture supplementation experiment (PSE). Lathosterol p values: a and b = 0.03.

Table 2: Sterol contents from milk produced by cows fed pasture supplementation experiment I diet: influence of feed rate

Sterol[#]	Feeding rate (kg/day)	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol	6	36	14.28	0.44	10.65	21.21
	8	36	14.46	0.38	10.41	18.06
	10	37	14.50	0.46	11.74	21.39
	12	40	14.28	0.32	11.29	18.71
	Average		14.38	0.20	10.41	21.39
Lathosterol	6	36	0.18	<0.02	0.09	0.34
	8	36	0.17	<0.02	0.08	0.30
	10	37	0.18	<0.02	0.10	0.27
	12	40	0.18	<0.02	0.09	0.27
	Average		0.18	<0.02	0.08	0.34
Campesterol	6	36	0.04	<0.02	<0.02	0.07
	8	36	0.04	<0.02	0.02	0.12
	10	37	0.04	<0.02	0.03	0.10
	12	40	0.04	<0.02	0.02	0.06
	Average		0.04	<0.02	<0.02	0.12
Lanosterol	6	36	0.23	<0.02	0.14	0.37
	8	36	0.24	<0.02	0.18	0.55
	10	37	0.21	<0.02	0.02	0.31
	12	40	0.22	<0.02	0.13	0.41
	Average		0.22	<0.02	0.02	0.55

[#]Total phytosterols, campestanol, stigmasterol, stigmastanol and β-sitosterol were less than the limit of reporting.

Table 3: Sterol contents from milk produced by cows fed the pasture supplementation experiment II diet: influence of feed type

Sterol[#]	Feeding regime*	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol	SPC	25	12.89	0.24	11.12	15.68
	PSEIIa	23	12.24[a]	0.13	11.19	13.07
	PSEIIb	15	13.07[b]	0.19	11.54	14.58
	Average		12.70	0.12	11.12	15.68
Lathosterol	SPC	25	0.148[a]	<0.02	0.08	0.28
	PSEIIa	23	0.126[b]	<0.02	0.09	0.17
	PSEIIb	15	0.126[b]	<0.02	0.08	0.16
	Average		0.135	<0.02	0.08	0.28
Campesterol	SPC	25	0.050[a]	<0.02	0.03	0.08
	PSEIIa	23	0.040[b]	<0.02	0.03	0.06
	PSEIIb	15	0.043	<0.02	0.03	0.05
	Average		0.045	<0.02	0.03	0.08
Lanosterol	SPC	25	0.17	<0.02	0.10	0.35
	PSEIIa	23	0.15[a]	<0.02	0.10	0.20
	PSEIIb	15	0.20[b]	<0.02	0.16	0.25
	Average		0.17	<0.02	0.10	0.35

[#]Total phytosterols, campestanol, stigmasterol, stigmastanol and β-sitosterol were less than the limit of reporting. For each type of sterol, means followed by different superscripts were significantly within the group and identified specifically after a post-hoc analysis between the superscripted treatment ($P < 0.05$). *Pasture supplementation experiment (PSE). Cholesterol p values: a and b = 0.02, Lathosterol p values: a = 0.03 and b = 0.07, campesterol p values: a and b = 0.00, lanosterol p values: a and b = 0.00.

Table 4: Sterol contents from milk produced by cows fed the pasture supplementation experiment II diet: influence of feed rate

Sterol[#]	Feeding rate (kg/day)	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol	8	12	12.50	0.26	11.22	13.99
	10	12	13.31	0.34	11.21	15.68
	12	21	12.69	0.18	11.12	14.02
	13.5	18	12.42	0.22	11.19	14.58
	Average		12.70	0.12	11.12	15.68
Lathosterol	8	12	0.12	<0.02	0.08	0.22
	10	12	0.15	<0.02	0.10	0.28
	12	21	0.13	<0.02	0.08	0.19
	13.5	18	0.14	<0.02	0.11	0.18
	Average		0.13	<0.02	0.08	0.28
Campesterol	8	12	0.05[a]	<0.02	0.04	0.07
	10	12	0.05	<0.02	0.03	0.08
	12	21	0.04	<0.02	0.03	0.07
	13.5	18	0.04[b]	<0.02	0.03	0.06
	Average		0.04	<0.02	0.03	0.08
Lanosterol	8	12	0.18	<0.02	0.14	0.30
	10	12	0.18	<0.02	0.14	0.35
	12	21	0.17	<0.02	0.13	0.25
	13.5	18	0.15	<0.02	0.10	0.23
	Average		0.17	<0.02	0.10	0.35

[#]Total phytosterols, campestanol, stigmasterol, stigmastanol and β-sitosterol were less than the limit of reporting. For each type of sterol, means followed by different superscripts were significantly within the group and identified specifically after a post-hoc analysis between the superscripted treatment ($P < 0.05$). Campesterol p values: a and b = 0.00.

Table 5: Sterol contents from milk produced by cows fed the tannin and cotton seed oil experiment diet

Sterol[#]	Feeding regime*	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol	Control	7	12.26	0.36	11.33	13.83
	CSO	8	12.60	0.62	10.40	15.93
	TANN	8	12.74	0.88	9.93	17.65
	TCSO	10	12.42	0.35	10.62	13.81
	Average		12.51	0.28	9.93	17.65
Lathosterol	Control	7	0.17	0.02	0.11	0.28
	CSO	8	0.17	0.03	0.07	0.36
	TANN	8	0.13	<0.02	0.07	0.22
	TCSO	10	0.15	0.02	0.09	0.27
	Average		0.15	<0.02	0.07	0.36
Lanosterol	Control	7	0.21	0.03	0.12	0.30
	CSO	8	0.18	0.03	0.05	0.30
	TANN	8	0.19	0.03	0.12	0.29
	TCSO	10	0.19	0.02	0.07	0.29
	Average		0.19	<0.02	0.05	0.30

#Total phytosterols, campestanol, campesterol, stigmasterol, stigmastanol and β-sitosterol were less than the limit of reporting. *Cotton seed oil (CSO), tannin (TANN), tannin and cotton seed oil (TCSO).

Table 6: Sterol contents from milk produced by cows fed the grape marc experiment diet

Sterols[#]	Feeding type*	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol	Control	25	16.47	0.54	11.99	22.67
	DGM	20	15.43	0.57	11.21	20.97
	WGM	21	16.46	0.59	11.77	23.77
	Total	66	16.15	0.33	11.21	23.77
Lathosterol	Control	25	0.11	<0.02	0.02	0.21
	DGM	20	0.12	<0.02	0.06	0.23
	WGM	21	0.13	<0.02	0.08	0.21
	Total	66	0.12	<0.02	0.02	0.23
Campesterol	Control	25	<0.02	<0.02	0.03	0.07
	DGM	20	<0.02	<0.02	0.01	0.07
	WGM	21	<0.02	<0.02	0.03	0.08
	Total	66	<0.02	<0.02	0.01	0.08
Lanosterol	Control	25	0.26[a]	<0.02	0.12	0.56
	DGM	20	0.18[b]	<0.02	0.06	0.35
	WGM	21	0.22	<0.02	0.12	0.37
	Total	66	0.22	<0.02	0.06	0.56

[#]Total phytosterols, campestanol, stigmasterol, stigmastanol and β-sitosterol were less than the limit of reporting. For each type of sterol, means followed by different superscripts were significantly within the group and identified specifically after a post-hoc analysis between the superscripted treatment ($P < 0.05$). *Dried grape marc (DGM), wet grape marc (WGM). Lanosterol p values: a and b = 0.003.

Table 7: Sterol contents from milk produced by cows fed the rumen protected experiment diet

Sterol	Feeding types*	N	Mean sterol content, mg/100mL	Std. Error	Min mg/100 mL	Max mg/100 mL
Cholesterol[a]	Control	7	12.96	0.28	11.71	13.73
	OP	1	13.66	N/A	13.66	13.66
	SP	3	10.09	0.21	9.68	10.31
	CSP	1	11.16	N/A	11.16	11.16
	Average		12.15	0.43	9.68	13.73
Lathosterol	Control	7	0.07	<0.02	0.06	0.09
	OP	1	0.11	N/A	0.11	0.11
	SP	3	0.06	<0.02	0.05	0.06
	CSP	1	0.05	N/A	0.05	0.05
	Average		0.07	<0.02	0.05	0.11
Campesterol	Control	7	0.04	<0.02	0.03	0.04
	OP	1	0.05	N/A	0.05	0.05
	SP	3	0.05	<0.02	0.04	0.06
	CSP	1	0.04	N/A	0.04	0.04
	Average		0.04	<0.02	0.03	0.06
Lanosterol	Control	7	0.19	<0.02	0.15	0.23
	OP	1	0.26	N/A	0.26	0.26
	SP	3	0.21	0.02	0.16	0.24
	CSP	1	0.11	N/A	0.11	0.11
	Average		0.19	<0.02	0.11	0.26

Total phytosterols, campestanol, stigmasterol, stigmastanol and β-sitosterol were less than the limit of reporting; all cholesterol concentrations were significantly different ($P < 0.05$). *Omega protected (OP), sterol protected (SP), canola soy protected (CSP). Cholesterol *p* values: a and b = 0.001.

Supplementary Material

Modification of the sterol profile in milk through feeding

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Feeding Regimes

The following text and tables provide additional details of the feeding regimes and compositions of the various experimental trials. In each of the experiments, representative samples of morning and afternoon milk were collected from individual cows over the last two days of the experiment using an inline meter (DeLaval International, Tumba, Sweden). Milk samples were combined and stored in opaque plastic containers that were later frozen at -20°C prior to analysis.

Pasture Supplementation Experiment I

In this experiment, a total of 216 cows were allocated into groups of seventy-two cows for three treatments: the standard practice control (SPCtrl), and two pasture supplementation groups (PSE1a and PSE1b). The cows were then allocated into two replicates within each group set. All cows were fed twice daily and supplements were given in addition to ryegrass pasture. The mode of feeding was different for the SPCtrl diet where the cows were given their supplements in the dairy and in the paddock, whereas the cows on the PSE1a and PSE1b diets were given feed supplements that were mixed in a wagon and placed in a concrete feed pad. The pasture allowance for each of the cows was 14 kg DM/day (to ground level). The groups were further divided into four groups of nine cows which were each fed 6, 8, 10, or 12 kg of supplement based on the DM content per day to evaluate the influence of feed amount. Table S1 presents the composition of the feed given to the cows in this regime.

Table S1: Pasture supplementation experiment I feed composition

Feed type	SPCtrl	PSE1a	PSE1b
Barley grain	75%	75%	25%
Maize grain			30%
Ryegrass silage	25%	25%	
Lucerne hay			25%
Maize silage			20%

% values are with respect to DM content

Pasture Supplementation Experiment II

In this experiment a total of 160 cows were allocated into three groups for three treatments: the SPCtrl (64 cows), and pasture supplementation groups (PSE2a and PSE2b) with 64 cows in PSE2a and 32 cows in PSE2b). The cows were then allocated into two replicates within each group set. All cows were fed twice daily and supplements were given in addition to ryegrass pasture. The mode of feeding was different for the SPCtrl diet where the cows were given their supplements in the dairy and in the paddock, whereas the cows on the PSE2a and PSE2b diets were given feed supplements that were prepared in a wagon and placed in a concrete feed pad. Cows on the SPCtrl and PSE2a groups were divided into four groups of eight cows (per replicate) which were each fed 8, 10, 12 or 14 kg of supplement based on the DM content per day whereas cows on the PSE2b treatment were divided into two groups eight cows (per replicate) and were each fed 12 and 14 kg of supplement based on the DM content per day. Table S2a and Table S2b present the composition of the feed given to the cows in this regime for both sets of experiments and the experimental design matrix to evaluate the feeding amount respectively.

Table S2a: Pasture supplementation experiment II feed composition

Feed type	SPCtrl	PSE2a	PSE2b
Crushed wheat grain	72%	39%	23%
Pasture silage	28%		
Crushed maize grain		20%	20%
Lucerne hay		9%	9%
Maize silage		32%	32%
Canola meal[#]			16%

[#]solvent extracted; % values are with respect to DM content

Table S2b: Pasture supplementation experiment II design with varying feed amounts

	SPCtrl								PSE2a								PSE2b			
Total number of cows	64								64								32			
Replicates	A				B				A				B				A			
Cows/replicate	32				32				32				32				16		16	
Cows/treatment	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Amount of feed per cow (kg DM/day)	8	10	12	14	8	10	12	14	8	10	12	14	8	10	12	14	12	14	12	14

(Auldist et al., 2014)

Tannin and Cotton Seed Oil Feeding Experiment

In the tannin (TANN), cotton seed oil (CSO) and mixed tannin & cotton seed oil (TCSO) trial, a total of ten cows were used for the experiment with two cows (donor) placed on the control for the first twelve out of the sixteen weeks of the experiment. These cows were set aside and were only used as a substitute during the experiment if cows one to eight were unable to complete their respective diets. In addition to their supplemented feed diets, all cows were also offered 6 kg DM of dairy concentrate (crushed wheat, canola meal, mineral mix and molasses powder), and approximately 20 kg DM of alfalfa. The experimental regime and feeding compositions are presented in Table S3a and Table S3b respectively.

Table S3a: Tannin and cotton seed oil feeding experiment regime

Cows	Week 1-4	Week 5-8	Week 9-12	Week 13-16
1	CSO	TANN	Control	TCSO
2	Control	CSO	TCSO	TANN
3	TANN	TCSO	CSO	Control
4	TCSO	Control	TANN	CSO
5	TANN	Control	CSO	TCSO
6	CSO	TANN	TCSO	Control
7	TCSO	CSO	Control	TANN
8	Control	TCSO	TANN	CSO
9	Control (donor)			
10	Control (donor)			

Table S3b: Tannin and cotton seed oil feeding experiment feed composition

Feed type	Control	CSO	TANN	TCSO
Crushed wheat/kg	4.1	4.1	4.1	4.1
Cold-pressed canola/kg	1.5	1.5	1.5	1.5
Mineral mix/kg	0.12	0.12	0.12	0.12
Palabind molasses powder/kg	0.28	0.28	0.28	0.28
Cotton seed oil/kg		0.8		0.8
Tannin/kg			0.4	0.4

Grape Marc Feeding Experiment

In the grape marc feeding experiment, a total of thirty-five cows were used and further subdivided into three feeding regimes with sixteen cows given the control feed, ten given the dried grape marc (DGM) feed, and nine given the ensiled grape marc (EGM). All cows were initially placed on the control diet for three weeks prior to being placed on their respective diets. In addition to the allocated diets, the cows were also offered alfalfa hay and a dairy concentrate mix (consisting of 93%, 4.7%, and 2.3% (DM) of crushed wheat, dried molasses, and mineral mix respectively). The experimental regime and feeding compositions are presented in Table S4a and Table S4b respectively.

Table S4a: Grape marc feeding experiment regime

Group	Week 1-3	Week 4-6
Control	Control	Control
DGM	Control	DGM
EGM	Control	EGM

Table S4b: Grape marc feeding experiment feed composition

Feed type	Control	DGM	EGM
Alfalfa hay kg	14	9	9
Crushed wheat/kg	6	1	1
Molasses/kg	0.2	0.2	0.2
Mineral and lucerne hay/kg	0.1	0.1	0.1
DGM/kg		5	
EGM/kg			5

Rumen Protected Feeding Experiment

In the rumen protected feeding experiment, a total of seven cows were used with six cows placed on the control diet for the first two weeks of the experiment. All cows were then placed on their respective omega protected (OP), sterol protected (SP), or the canola/soybean protected (CSP) diets from week three onwards. Samples were collected at the end of the second and fourth weeks of the experiment. On the sampling day, the cows were milked twice (morning and afternoon) with both samples combined to obtain a single daily sample from each cow. The experimental regime is presented in Table S5.

Table S5: Rumen protected feeding experiment regime

Cow	Week 1	Week 2	Week 3	Week 4
1	Control	Control	OP	OP
2	Control	Control	OP	OP
3	Control	Control	SP	SP
4	Control	Control	SP	SP
5	Control	Control	Control	Control
6	Control	Control	Control	Control
7			CSP	CSP