Accumulation of Citrus Flavanones in Bovine Milk Following Citrus Pulp Incorporation into the Diet of Dairy Cows

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Abstract

This study was focus on the dairy polyphenols. Previous studies have found polyphenols in different types of milk and cheese. However, there is no evidence for the phenolic metabolism from the diet to the final product through livestock. The aim of this project was to understand the phenolic metabolism in dairy cows by introducing high phenolic diet to cows with selected ingredients from the industrial waste of food and ultimately improving the public health by introducing the polyphenol-rich milk to consumers.

To understand the phenolic metabolism in cows and to explore the feasibility of enriching bovine milk with polyphenols through the incorporation of citrus pulp- a food industrial waste, into the ruminant feeding system. In this study, 8 lactating Holstein fistulated cows were enrolled on to the trial. The cows were fed either a standard farm diet (control group) or the standard feed with 4 kg/day/ head of citrus pulp (containing 193.2 mg/4 kg of hesperetin) and 1kg/day/head of soya meal (treatment group) for 4 weeks. Milk was collected twice daily, three times per week and analysed for milk yield, macronutrient composition, somatic cell count and the presence of polyphenols/flavonoids.

Our research suggests that the incorporation of flavonoids such as hesperetin into ruminant milk is possible through traditional animal feeding. Using our design, feeding cows with citrus pulp increased the flavanone content of milk to 0.40 mg/100 mL hesperetin of milk, with other phenolics at lower amounts: 0.03 mg vanillic acid, 0.06mg isoferulic acid, 0.03 mg 3,4-hydroxybenzoic acid, 0.03 mg 4-hydroxybenzoic acid, 0.07 mg salicylic acid, 0.09 mg Ferulic acid and 0.03 mg daidzein per 100 mL. The data results may represent a novel means by which to increase regular consumption of flavonoids in humans with potential health benefits.

Keywords: Polyphenols; Dry Matter; Citrus; Hesperetin; Somatic Cell Count

Abbreviations

DM: Dry Matter; TMR: Total Mixed Rations; STD: Standard Deviation; SEM: Standard Error of the Mean; SCC: Somatic Cell Count; EC: Multi-Electrode Detector; HPLC: High Performance Chromatography; CVD: Cardiovascular Disease; CHD: Coronary Heart Disease; AD: Alzheimer's Disease; PD: Parkinson's Disease; RT: Retention Time

Introduction

Polyphenols are a group of plant secondary metabolites, which are commonly consumed in the human diet and have been postulated to promote human health [1-3]. Two relatively rich sources of polyphenols are citrus [4,5] and soya [5,6], which contain high levels of flavanones and isoflavones respectively. Evidence suggests that polyphenols/flavonoids may interact with, and attenuate, pathological pathways involved in the progression of chronic diseases such as cardiovascular disease [7-10], neuronal degeneration [11-15] and cancer [16,17]. It is well reported that polyphenols/flavonoids and their metabolites enter the circulation following their dietary intake where

they may exert influence on vascular homeostasis [18,19]. However, despite their wide occurrence in plant foods, it remains a challenge to increase the human consumption of these beneficial dietary components. Current low levels of fruit and vegetable consumption, and/or consumption of beverages derived from plant origin (juices, coffee, tea etc.) [20], indicate that intake sufficient to raise circulating flavonoids/metabolites to meaningful physiological levels remains a challenge [2]. Thus, there is an interest in developing novel, economically viable dietary strategies to increase flavonoid consumption in individuals and at the population level.

It is well reported that flavonoids enter the circulation, where they may be distributed to various tissues and/or be renally excreted. An additional fate of absorbed flavonoids is the mammary gland, where they have been shown to be incorporated into the milk of both lactating women and animals [21,22]. A number of phytochemicals have been detected in dairy products, including caffeic acid, chlorogenic acid and ferulic acid [21,23,24]. For example, quercetin has been detected in goat's milk/cheese following summer grazing on a wild forb mixture [23]. With respect to humans, epicatechin, epicatechin gallate, epigallocatechin gallate, naringenin, kaempferol, hesperetin, quercetin, p-hydroxybenzoic acid and ferulic acid have been detected in the milk of lactating women following their habitual diet (i.e. no polyphenol supplementation) [21,24]. The nutrient composition of milk is affected by a number of factors, including season, milking time [25] and animal diet [26]. There are a few ingredients from food industrial waste, for example pomegranate peel, green tea waste and citrus pulp, which have been incorporated into animal feed. Although these ingredients are industrial by-products, in general they are a rich source of energy/nutrients with good economic value. Especially citrus pulp which was introduced into animal feed in early 1911 and commercially processed as animal feed in recent decades [27]. Previous studies have investigated the impact of introducing various food processing waste stream products, such as citrus pulp [28,29], pomegranate peel [30,31], green tea waste [32], tomato and cucumber waste [33] on the composition of milk. Notably, incorporation of a distillate of rosemary leaves into the feed of goats resulted in the incorporation of naringin, hesperidin, genkwanin and the phenolic acids gallic acid and carnosic acid into goat's milk [34]. Furthermore, rosmarinic acid and carnosic acid were also detected in the plasma of suckling goat kids of the mothers fed the rosemary extract.

Although the phenolic content is significantly lower in dairy products compared to that of fruit and vegetables, milk is an important source of micronutrients and macronutrients and is commonly consumed at high levels across all age groups (UK approximately 200 g/ per day on average) [20]. As such, incorporation of moderate levels of flavonoids/polyphenols into milk and dairy products through the feeding of animals with cheap food waste rich in these phytochemicals, may provide a novel way to increase polyphenol intake, particularly for people with a low fruit and vegetable intake. In the current study, we investigate the extent to which flavanones are incorporated into the milk of dairy cows fed citrus pulp as part of their regular lactating farm feed.

Materials and Method

Reagents

3,4-hydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, ferulic acid, isoferulic acid, naringin, hesperetin, salicylic acid, daidzein, quceretin, hexane, hydrochloric acid (HCl), acetonitrile, 1,1,1,3,3,3-Hexafluoro-2-propanol, ethyl acetate and β-glucuronidase were obtained from Sigma-Aldrich (Dorset, UK). Genestic acid and chlorogenic acid were purchased from Extrasynthese (Lyon, France). Methanol and water were purchased from Fischer Scientific (Loughborough, UK). Formic acid was obtained from Riedel-deHaën (Seelze, Germany). 10 mL syringe was supplied by BD Plastipak (Madrid, Spain), 0.8x 40 mm needles were purchased from Terumo (Surrey, UK). Ministar single–use filter units (0.45 μm) was obtained from Sartorius Stedim (Surrey, UK). Spectrophotometer or GENios Pro microplate reader (Tecan UK Ltd., Theale, UK) with Magellan software (Tecan, UK). Luna 3u C18 (2) 100 Å (size: 150 x 4.60) Colum was obtained from Phenomenex (Cheshire, UK).

Cows and management

Eight multiparous dairy cows (Holstein-Friesian) in mid-lactation, averaging 29.6 ± 4.6 kg milk/day at the start of the study were used. Four cows were randomly assigned to a 'control' group (No.808, No.860, No.1000 and No.1013) and were housed in a cubicle yard with rubber filled mattress and wood shavings as additional bedding. Four cows assigned as 'treatment' group (No.980, No.989, No.1003 and

No.1058) and were housed similarly. All cows had free access to clean water via a trough system and were milked in a conventional her-ringbone parlour twice daily at approximately 06:00 and 16:00 h. All procedures were ethically assessed and cleared prior to licencing and monitoring was conducted by the UK Home Office under the auspices of the Animal Scientific Procedures Act (1986) [35].

Animal diets

Control and treatment animals were fed total mixed ration (TMR) the composition of which are given in Table 1. The treatment diet contained 180 g/kg dry matter (DM) citrus pulp (Mole Valley Farmers Ltd, Devon, UK) and an additional 45 g/kg DM soya bean meal (KW Agriculture Ltd, Hampshire, UK) to ensure that diets were iso-nitrogenous (Table 1). No other adjustments to nutrient composition were made between the two diets. Diets were available to cows *ad-libitum* with diets being provided once daily in the morning with a target of 10% refusals. Refusals were removed and weighed the following morning prior to the next feeding. Diet components were sampled daily and bulked to provide composite samples. The DM content of the offered feeds was determined by oven drying at 100°C for 23 hours and the DM content of uneaten feed (refusals) was determined by drying at 60°C for 48 hours. Diet intake (DM) was calculated by difference between that offered and refused. Samples of undried offered diet were also stored at -20°C for subsequent chemical analysis.

Ingredients	Control	Treatment
Maize silage	280.4	217.3
Perennial ryegrass silage	61.8	47.9
Italian ryegrass silage	143.8	111.5
Hay	34.5	26.7
Straw	17.5	13.6
Concentrate blend	442.0	342.4
Fat	11.8	9.2
Acid buffer	4.1	3.2
Salt	4.1	3.2
Citrus pulp	-	180.0
Soya bean meal	-	45.0

Table 1: Diet composition (g/kg DM): control diet or a treatment diet containing citrus pulp and additional soya bean meal.

The control and treatment diets (TMR) were dried, milled (screen size: 1 mm) and extracted for polyphenol analysis. 1.5 g of dried, milled material was mixed with 10 ml of 0.1M HCl in methanol, votexed for 1 minute and sonicated for a further 5 minutes at room tem-perature. The mixture was centrifuged for 15 minutes at 4000x g at room temperature and 3 mL of the supernatant retained and mixed with 3 mL 5M HCl for 1h at 90°C to hydrolyse flavonoid glycosidic linkages. Hydrolysates were placed on ice to cool before drying under vacuum. The dried extracts were dissolved in 1 ml of the mobile phase A (95% of HPLC grade water, 5% of methanol and 0.2% of formic acid) and passed through a 0.22 μm filter before analysis. The phenolic content of two diets were analysed and quantified prior to the feeding study, see Flavonoid/polyphenol analysis for the details.

The concentrate blend containing on a fresh weight basis (g/1000g): wheat 260g, rapeseed meal (00) 175g, soya bean meal (Hipro) 160g, soyabean hulls 120g, sugar beet pulp 100g, wheat distillers 100g, molasses 40g, minerals 20g, protected fat (85%) 15g, urea (2000) 10g was formulated to provide DM 887 g/1000g and on a DM basis ME 12.9 MJ/kg, crude protein 274, oil 45, NDF 250, starch 187, sugar 73, ash 76, Ca 10, P 6.5, Mg 4.2.

Milk yield and macronutrient composition

Milk yields (kg/day) were recorded daily during each milking session. Fat, protein and lactose concentration were assessed in milk harvested from each cow, twice a day on three separate days. Milk samples were preserved with potassium dichromate, (1 mg/ml, Lactabs,

Thompson and Capper, Runcorn, UK) and stored at 4°C until macronutrient analysis using near mid-infrared spectroscopy (Milkoscan FT6000 infrared analyser, Foss Ltd., Warrington, UK). A further milk sample (30 ml) was retained from each milking and stored frozen (-20°C) until analysed for polyphenol content.

Flavonoid/Polyphenol Analysis

The milk was extracted as previously detailed [21] with minor modification. Briefly, milks collected over the day were combined and 20 ml of hexane was mixed with 15 ml of each milk sample for defatting, followed by centrifugation for 10 minutes at 4000x g. This was repeated 3 times and the supernatants retained. HCl (0.5 M) was added to fat free milk samples for deproteinisation, followed by centrifugation for 10 minutes at 4000 x g. After deproteinisation, the pH of each sample was adjusted to pH 4.5 with NaOH (0.1 M) prior to addition of 50 µl β-glucuronidase for 45 minutes at 37°C. After enzymatic hydrolysis, milk polyphenols were extracted using 1 ml of ethyl acetate (0.902 g/ml) three times with the upper layer liquid collected each time and dried under vacuum. The dried extracts were dissolved in 1ml of the mobile phase A and kept at -80°C until analysis.

Extracts from milk samples and feeds were resolved and quantified using an Agilent HPLC 1100 series equipped with a quaternary pump, autosampler, column and sample thermostat linked to UV/Vis, fluorescence, electrochemical detectors (EC) (Model 5600 Å, equipped with a Cell Model 6210 with an array of four electrodes). Chromatography was based on a Phenomenex Luna C18 (2) column (150 × 4.6 mm, 3-µm particle size) with guard column. The mobile phase consisted of 5% methanol and 0.2% formic acid in HPLC grade water (phase A), whilst phase B consisted of 50% acetonitrile and 0.2% formic acid in water. The following gradient protocol was run: 0-5 min, 95% A, 5% B; 50 min, 50% A, 50% B; 65 min, 0% A, 100% B; 69 min, 0% A; 100% B; 70 min, 95% A, 5% B. The flow rate was 0.7 ml/min.

Statistical Analysis

IBM SPSS Statistic Data Editor (Version 21) was used for all statistics. Milk yield, milk composition and somatic cell count for weeks one to four were statistically analysed by the GLM repeated measures procedure of IBM SPSS Statistics 19. Preliminary measurements of milk yield were made for all cows over two weeks prior to the start of citrus feeding and these data were included in the model to adjust measurements of milk production response after the treatments were imposed. Data on the animal feeds and milk polyphenols were subjected to paired samples T testorone-way analysis of variance (ANOVA) followed by Tukey's posthoc test with 0.05 significance level. The data were reported as means ± standards deviations of three replicates. Differences at P < 0.05 were considered statistically significant.

Results and Discussion

Polyphenol composition and intake of feeds

9 polyphenols were identified in the control diet, whereas 11 were detected in the treatment diet. The quantification of feeds indicated that flavanones were the main flavonoid subgroups detectable in citrus pulp (Table 2). In the treatment diet: TMR mixed with 18% of citrus pulp and 4.5% of soya meal enrich the polyphenols profile (3,4-hydroxybenzoic acid and naringenin) compared to the TMR control diet.

The phenolic data on the animal feeds were subjected to one-way ANOVA followed by Tukey's posthoc test with 0.05 significance level. The data (n = 3) were reported as means ± standards deviations of three replicates (*** P < 0.001, ** P < 0.01 and * P < 0.05).

Polyphenol (mg/22.2kg)	Control Diet	Treatment Diet	P Value
Vanillic acid	256.92 ± 10.70	256.62 ± 11.54	0.39
3,4-hydroxybenzoic acid	-	152.29 ± 3.32	-
4-hydroxybenzoic acid	129.05 ± 5.0	132.74 ± 0.54	0.27
Syringic acid	73.22 ± 4.68	78061 ± 1.15	0.125
Daidzein	183.45 ± 5.64	148.09 ± 2.0	0.001***
Naringenin	-	47.43 ± 2.48	-
Ferulic acid	94.48 ± 2.0	104.0 ± 2.04	0.004**
Salicylic acid	632.45 ± 2.44	551.30 ± 2.56	0***
Isoferulic acid	20.89 ± 1.26	19.71 ± 0.47	0.2
Caffeic acid	86.94 ± 1.26	281.33 ± 1.86	0***
Hesperetin	2464.64 ± 214.1	3071.21 ± 199.44	0.023*

Table 2: Polyphenol profile of TMR diet (control) and treatment diet (TMR plus soya/citrus).

Milk yield and macronutrient content

Average milk yield per day along with standard error (SEM) is presented in Table 3. Additionally, the somatic cell count was less than 200,000 which indicates the absence of mammary gland infection [36]. There were no significant differences in the milk yield, macronutrient levels, milk fat composition (Table 4) and somatic cell count (SCC) between control (TMR) and treatment group (TMR + citrus). Although there was found to be no significant difference in mean SCC between the control and the citrus fed groups, the cell count for the citrus group appeared to be much smaller than for the control grouper (Table 3). Moreover, there was a significant difference in the SCC between citrus feeding period and post citrus feeding period in two cows (No. 989 and 1058) from the treatment group (Figure 1). The phenolic transfer efficiency rates are reported in Table 5. The transfer efficiency rate was significantly greater in the treatment group (cows fed citrus pulp) compared to the control group for three phenolic compounds: ferulic acid, isoferulic acid and hesperetin.

	Control	Treatment	SEM	P value
Milk yield (kg/day)	29.25	26.91	1.03	0.19
Milk components (g/day)				
Fat	1106.00	1077.00	54.90	0.74
Protein	933.00	885.00	29.20	0.31
Lactose	1260.00	1163.00	50.60	0.25
Milk composition (g/kg)				
Fat	37.96	39.85	1.23	0.34
Protein	32.08	32.94	0.78	0.40
Lactose	43.01	43.09	0.09	0.96
*SCC (cells/ml)	661.00	137.00	208.70	0.15

Table 3: Milk yield milk composition and SCC in cows fed a control diet or a diet containing citrus pulp and soya (all SCC values are x 1000).

* Milk somatic cell count (SCC) is an indirect measurement of monitoring mammary gland infection (↑cells count = ↑infection).
Data are expressed as the mean ± STD (n = 4).

%	MUFA	PUFA	SFA	UFA
Control	26.06 ± 2.30	3.51 ± 0.41	65.21 ± 1.85	34.43 ± 2.09
Treatment	26.0 ± 2.85	3.48 ± 0.57	66.26 ± 1.97	33.31 ± 2.10
P value	0.935	0.861	0.133	0.143

Table 4: Milk fat composition in cows fed a control diet or a diet containing citrus pulp and soya.

The milk fat composition including: monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA), saturated fatty acid (SFA) and unsaturated fatty acid (UFA). The statistical data were subjected to one-way ANOVA followed by Tukey's posthoc test with 0.05 significance level. The data (n = 4) were reported as means % ± standards deviations of three replicates (*** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$)

Transfer efficiency %	Control	Citrus	*P* Value
Vanillic acid	2.74 ± 0.18	2.83 ± 0.72	0.48
3-4 hydroxybenzoic acid	-	6.40 ± 0.35	-
4 hydroxybenzoic acid	8.47 ± 0.42	6.63 ± 0.16	0.002*
Daidzein	4.00 ± 0.10	6.04 ± 0.16	0.000***
Ferulic acid	26.16 ± 0.48	23.83 ± 0.33	0.001***
Salicylic acid	2.53 ± 0.49	2.83 ± 0.63	0.36
Isoferulic acid	86.10 ± 1.85	92.15 ± 0.76	0.006*
Hesperetin	0.42 ± 0.02	7.98 ± 0.43	0.000***

Table 5: Transfer efficiency rate of polyphenols from animal feed to milk.

*Calculation: (phenolic compounds in the milk / phenolic compounds in the treatment diet) *100.The statistical data on the phenolic compounds were subjected to one-way ANOVA followed by Tukey's posthoc test with 0.05 significance level. The data were reported as means ± standards deviations of three replicates (*** P< 0.001, ** P< 0.01 and * P< 0.05).*
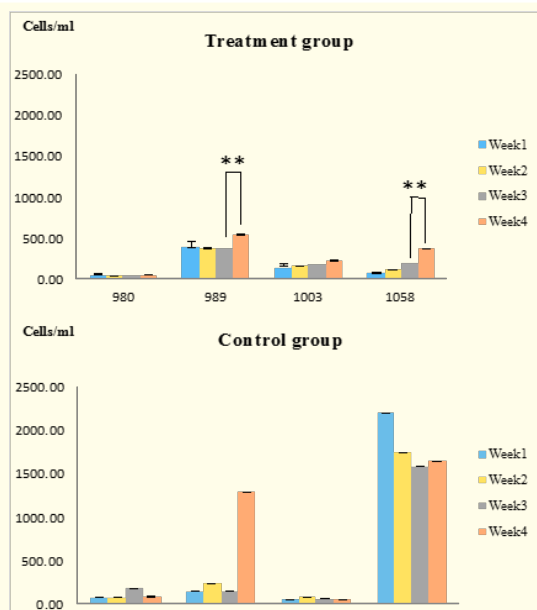

Figure 1: Milk somatic cell count (SCC): the comparison between the control and the treatment group.

The citrus fed group (Cow's No.980, No.989, No.1003 and No.1058) and the control group (Cow's No.808, No.860, No.1000 and No.1013), the week 1-3 were citrus feeding period in the treatment group, the week 4 was post citrus feeding period and the diet in this week was the same diet as control group. (No.989 P = 0.001, No. 1058 P = 0.001). Milk somatic cell count (SCC) is an indirect measurement of monitoring mammary gland infection (↑cells count = ↑ infection). Data are expressed as the mean ± STD (n = 3) and the P value was generated from paired samples T test (*** P < 0.001, ** P < 0.01 and * P < 0.05).

Milk polyphenols

The polyphenol content of milk samples was identified using a HPLC multi-electrode detector system (see Figure 2 for example chromatograms).Nine polyphenols with different retention times (RT) were identified in samples from the treatment group: including 3,4-hydroxybenzoic acid (RT: 14.5 min), 4-hydroxybenzoic acid (RT: 22 min), vanillic acid (RT: 26 min), caffeic acid (RT: 27.5 min), ferulic acid (RT: 39 min),isoferulic acid (RT: 41 min), hesperetin, (RT: 47 min)(l) salicylic acid (RT: 48.5 min) and daidzen (RT: 55.5 min). Analysis of the milk samples from the control group identified eight phenolic compounds with the same retention time; Hesperetin was not detected in these samples. Additionally, the quantification of the main phenolic compounds in the milk samples was calculated as mean ± standard deviation and presented in four charts (Figure 2). The level of hesperetin (the minor signal of hesperetin was normal on the by electrochemical detection) was found to be significantly higher in samples from the treatment group compared to samples from the control group throughout 4 weeks of citrus feeding. There were also found to be significantly higher levels of daidzein in the samples from the treatment group compared to the samples from the control group at week 2 and week 3.
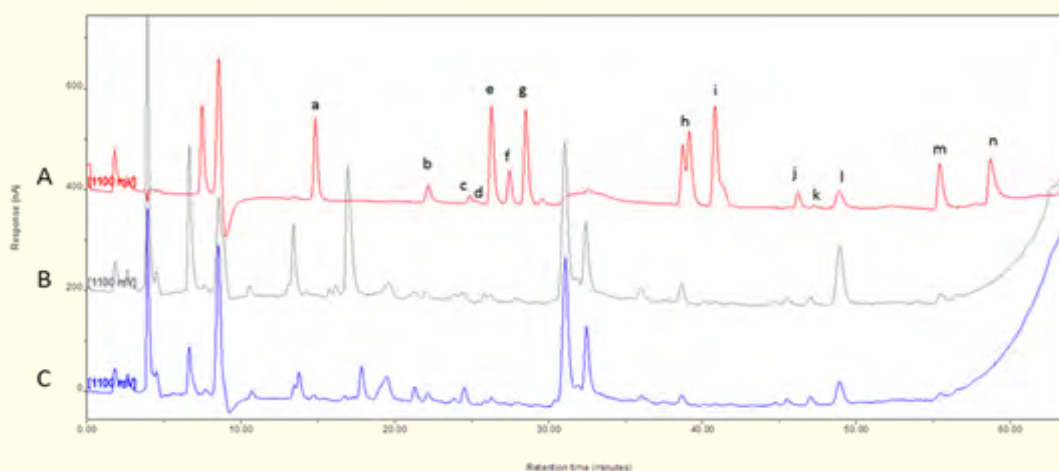

Figure 2: *Typical HPLC chromatograms of extracted milk samples detected by electrochemical detection (EDC): an Agilent HPLC 1100 series equipped with a quaternary pump, autosampler, column and sample thermostat linked to UV/Vis, fluorescence, electrochemical detectors (Model 5600A, equipped with a Cell Model 6210 with an array of four electrodes). Chromatography was based on a Phenomenex Luna C18 (2) column (150 × 4.6 mm, 3-μm particle size) with guard column. The following gradient protocol was run: 0-5 min, 95% A, 5% B; 50 min, 50% A, 50% B; 65 min, 0% A, 100% B; 69 min, 0% A; 100% B; 70 min, 95% A, 5% B. The flow rate was 0.7 ml/min. (A): Standards, (B): Citrus/ treatment group, (C): Control group. The standards including: (a) 3,4-hydroxybenzoic acid (b) 4-hydroxybenzoic acid (c) genestic acid (d) chlorogenic acid (e) vanillic acid (f) caffeic acid (g) syringic acid (h) ferulic acid (i) isoferulic acid (j)naringenin(k) hesperetin (l) salicylic acid (m) daidzein (n) quercetin.*
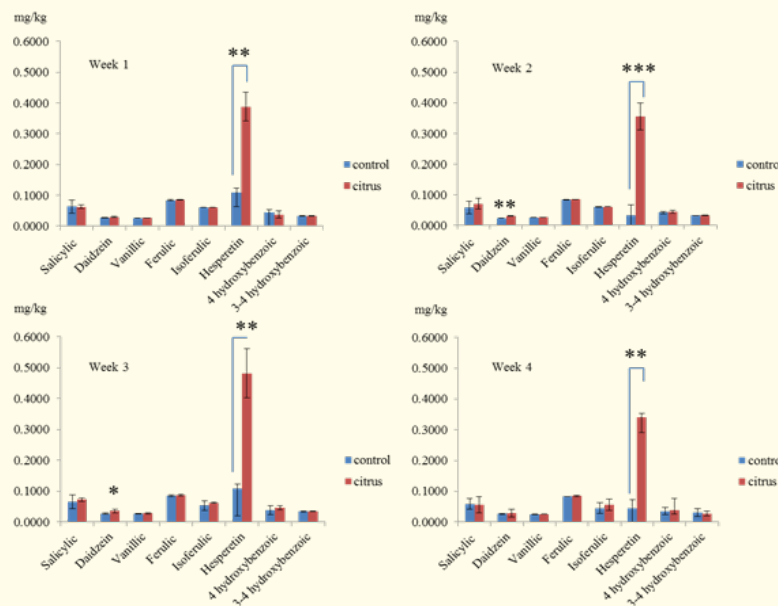
Figure 3: *Comparison of control group and treatment group on milk polyphenol profile from week 1 to week 4: eight polyphenols were detected in the milk and significant levels of hesperetin were found throughoutcitrus feeding trial as well as significant level of daidzein in week 2 and week 3. Data were expressed as the mean ± STD (n = 4).The statistical data on the phenolic compounds were subjected to one-way ANOVA followed by Tukey's posthoc test with 0.05 significance level. The datawere reported as means ± standards deviations of three replicates (*** P< 0.001, ** P< 0.01 and * P< 0.05).*

Discussion

Evidence suggests that polyphenols/flavonoids can be obtained from the daily diet and that these phytochemicals could help to combat certain chronic diseases [7,37,38]. However, despite their abundance in the diet, current low levels of fruit and vegetable consumption, and consumption of beverages derived from plant origin (e.g. juices, coffee and tea) [20], indicate that intake sufficient to raise circulating metabolites to meaningful physiological levels remains a challenge [2]. The main purpose of the present feeding trial was to explore the feasibility of incorporating flavanones into milk by supplementing bovine feed with citrus pulp. After 4 weeks of feeding, the citrus pulp/soya meal intervention did not affect milk yield and/or macronutrient status of milk (Table 3). This is in agreement with previous findings that report no significant effects on milk yields following supplementation of animal feeds with flavonoid rich rosemary [34] or green tea [39]. In contrast, other polyphenol-containing industrial waste products, such as pomegranate peel extract [31] and soybean hulls [40], have been reported to increase milk yield in both dairy cows and goats. Furthermore, 4 weeks of feeding with the citrus supplemented feed led to a reduction in the somatic cell count (SCC) in two of the citrus fed cows. SCC is an indirect measure of mammary infection caused by frequent milking, indicating that infection levels were more stable in the citrus supplemented animals, however, there was no significant different of SCC on the mean of all animals in the citrus fed group (Table 3).

A number of phenolic compounds were identified in the milk derived from both control and citrus supplemented animals which included: 3,4-hydroxybenzoic acid, 4-hydroxybenzoic acid, vanillic acid, ferulic acid, isoferulic acid, hesperetin, salicylic acid and daidzein (Figure 3). Relative to the control group, the milk obtained from citrus/soya supplemented cows had significantly higher levels of hesperetin at all time points measured. Additionally, the transfer efficiency (Table 5) was calculated to obtain a further information of the polyphenol metabolism between animal feed and the final product (i.e. milk). A number of polyphenols were detected in bovine milk of both experimental groups over the course of the feeding period. These may have their origin in the TMR feed and/or may be gener-

ated from other phenolics and amino acids [41] present in the feed. With regards to the latter, 3,4-hydroxybenzoic acid was absent in the regular lactating diet (Table 2) but appeared in milk from cows fed the regular diet without supplementation (Figure 3). Our data reflect the findings of previous studies reporting the presence of polyphenols in milk after feeding polyphenol rich diets to lactating animals [22,34,39,42], although, to our knowledge, this is the first time hesperetin and salicylic acid, in addition to diadzein, ferulic acid, isoferulic acid, hesperetin, 4-hydroxybenzoic acids, 3,4-hydroxybenzoic acid, vanillic acid and isoferulic acid have been detected in the milk of animals fed a standard lactating farm feed (TMR). Naringenin, syringic acid and caffeic acid were not detected in milk samples of either group (Figure 3) despite their presence in the TMR feed (Table 2). An elevated amount of daidzein was measured at week 2 and week 3 in the citrus supplemented group, despite control animals receiving around the same level of daidzein intake from the TMR diet. It is possible that citrus pulp in the treatment diet improved the feed digestibility by helping cows absorb more daidzein in their gastric tract, as has been previously observed [43].

Our data are in agreement with a previous study [43] which reported that the addition of 18% citrus pulp to cow's diet leads to an improvement the feed digestibility and an increase in the phenolic concentrations in milk. In the current study, feeding cows with citrus pulp increased the flavanone content of milk to 0.391 mg flavonones (predominantly hesperetin) per 100 ml of milk, with other phenolics at lower amounts: 0.027 mgvanillic acid, 0.06mg isoferulic acid, 0.032mg 3,4-hydroxybenzoic acid, 0.316 mg 4-hydroxybenzoic acid, 0.07 mg salicylic acid, 0.09 mg ferulic acid and 0.03 mg daidzein per 100 ml. Although the polyphenol content detected in bovine milk is relatively low compared to that present in fruits and vegetables, the polyphenols in milk that originate from the diet of dairy cattle; through their digested system with ruminal fermentation; potentially have a higher absorption rate. Moreover, according to the National Diet and Nutrition Survey [20]: an average 200 ml of cow's milk was consumed per day in all age groups in the UK, the phenolic enriched milk from present study can provide 0.78 mg of hesperetin and 0.18 mg of salicylic acid, which may potentially have beneficial effects on maintaining cardiovascular health [44,45].

The citrus feeding study provides evidence for incorporation of flavanones into bovine milk following feeding with citrus pulp (industry waste product) for four weeks. This incorporation of flavanones into the animal feed led to accumulation of low levels of flavanones in milk. Increasing the flavanone content of in milk is likely to play a role in combating mammary gland inflammations [46] by exerting potential antimicrobial properties on gastrointestinal pathogens [47,48] resulting from pasture contamination [28], potentially reducing illness in livestock and increasing the shelf-life of milk [43], rather than contributing to daily polyphenol intake in a significant manner.

Acknowledgements

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