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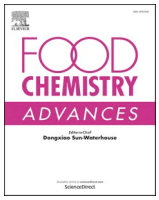
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# Impact of pasture feeding cows and seasonality on the concentration of selected fat-soluble compounds in milk and their ability to be used as biomarkers of "Grass-Fed" dairy

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ABSTRACT

This study investigated the impact of proportion of pasture in the cow diet on the concentration of selected fat-soluble compounds in milk including β-carotene, lutein, retinol (vitamin A), α-tocopherol (vitamin E) and zeaxanthin. Bulk tank milk samples ($n = 102$) were collected weekly ($n = 34$) across an entire lactation from three herds operating either a high (GRS), medium (PMR) or no pasture allowance (TMR) feeding system. Concentrations of β-carotene and lutein increased significantly with increasing pasture allowance, with $a > 2$ fold increase of β-carotene in milk, between high and no pasture allowance treatments. GRS feeding system resulted in milks with higher concentrations of retinol, α-tocopherol, and zeaxanthin, compared to both PMR and TMR systems. A biomarker model based on the fat-soluble compounds concentration in milk, demonstrated excellent differentiation of high pasture allowance derived milks from that of medium and no pasture milks, with area under the curve scores of 0.955 and 0.971 respectively. Strong correlations were observed between the concentrations of lutein and zeaxanthin in milk and the milks corresponding yellow colour, while a significant correlation was exhibited between β-carotene and b*-value. Such correlations with milk colour may have a significant impact on the consumer preference of milk and dairy products produced therefrom.

## 1. Introduction

Despite indoor-based dairy production systems being the most prevalent form of milk production globally, bovine feeding systems utilizing outdoor grazing or partial outdoor grazing have been demonstrated to have a positive effect on milk and dairy product composition, functional and sensory properties (Benbrook et al., 2018; McGuinness et al., 2024; Timlin et al., 2024, 2023). In addition to this, the more natural environment provided to the animals operating within these pasture fed systems, have resulted in an increased consumer demand for "Grass-Fed" dairy products on international markets (Kilcawley et al., 2018) and resulted in the development of 'Grass-Fed' standards to increase consumer confidence. While not ubiquitous across the world, these standards typically stipulate minimum time at pasture and minimum grass allowance which can vary between regions (Bord Bia, 2019; Morrisey, 2018; NZMP, 2022). The influence of pasture feeding systems on the fat-soluble nutrient content of milk and dairy products are however, less studied and require further exploration, particularly with respect to animal stage of lactation and season. Previous research has identified significant increases of fat-soluble nutrients in dairy products due to the inclusion of pasture in the feeding system including; higher *trans*-β-carotene concentrations in butter and cheese (O'Callaghan et al.,

*Abbreviations:* GRS, grass; PMR, partial mixed ration; TMR, total mixed ration; SOL, stage of lactation; ROC, receiver operating characteristic; PLSDA, partial least squares discriminatory analysis; AUC, area under the curve.

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2016, 2017), higher *trans*-β-carotene, α-tocopherol and retinol in milk (Mitani et al., 2021) and higher *trans*-β-carotene, α-tocopherol, lutein and retinol in milk (Şanta et al., 2022). Despite these findings, there is still limited information available on how these nutrients can vary in pasture based feeding systems across the season and lactation which have been previously demonstrated to have a significant impact on a range of milk components (Auldist et al., 1998; Gulati et al., 2018; Timlin et al., 2023).

Fat-soluble nutrients in milk such as β-carotene and other carotenoids are beneficial to the consumer as provitamin precursors, and therefore, have the potential to confer a nutritive value (Nagao, 2004). As efficient dietary antioxidants, carotenoids have been widely studied in the human diet, with increased consumption being indicated to support health including a reduced risk of cardiovascular disease, age-related macular degeneration and certain types of cancer (Cooperstone & Schwartz, 2024). As carotenoids cannot be synthesised in the body, their presence in milk is directly as a result of the animal's dietary intake of carotenoids. Considering larger ruminants such as cows have a lower digestive efficiency of carotenoids compared to other animals (Nozière et al., 2006), the carotenoid concentration and resulting characteristics of bovine milk is of particular reliance on their carotenoid intake. Not only do carotenoids contribute to the nutritive value of milk, they also confer a yellow/orange colour to milk and dairy products containing fat. As such, the carotenoid concentrations in milk may be used as potential biomarkers to differentiate 'Grass-Fed' and pasture derived dairy products.

Among multiple instrumental platforms capable of analysing the fat-soluble nutrients within milk, High performance liquid chromatography (HPLC) is known for its reproducibility in qualitative as well as quantitative measurements. HPLC hyphenated with Mass Spectrometry (HPLC-MS) can provide structural information of the compounds and is highly sensitive in that it can detect at levels as low as picogram to femtogram. Spectroscopy techniques are increasingly becoming adapted as a rapid method to analyse various micro components in milk, with one such study examining the carotenoid and vitamin concentrations in milk using near-infrared, mid-infrared and molecular fluorescence. However, the use of frozen samples in that study is likely to have reduced the prediction accuracy of such techniques (Soulat et al., 2020). We selected HPLC in this research owing to its suitability as an instrumental platform for routine analysis.

The objective of this study was to comprehensively examine the impact of increasing pasture allowance and stage of lactation on the fat-soluble nutrient content of milk using commonly operated global feeding systems including that of a high pasture allowance (commonly operated in temperate regions such as Ireland and New Zealand), a partial pasture allowance feed and a no pasture allowance (conventional indoor feeding system). This work further aimed to examine the use of milk fat-soluble nutrients as biomarkers of pasture-fed dairy.

## 2. Materials and methods

### 2.1. Chemicals & reagents

The chemical standards of β-carotene, lutein, and zeaxanthin were purchased from DHI Lab Products (Hørsholm, Denmark). Retinol, α-tocopherol, and trans- β-Apo-8′-carotenal were purchased from Merck (Darmstadt, Germany). All the solvents used were of high-performance liquid chromatography grade purchased from Merck (Darmstadt, Germany).

### 2.2. Experimental design and sample collection

Milks used in this study were collected as part of a broader comprehensive study previously described by Timlin et al. (2023) between months March and October. Briefly, bulk milk samples were collected weekly from three herds consuming one of high (grass; GRS) medium (PMR) and no (TMR) pasture allowance diets. The GRS herd were maintained outdoors and grazed a predominantly perennial ryegrass pasture (*Lolium perenne* L.) through rotational grazing (95 % annual average) and supplemented with concentrates (5 % annual average), the no pasture allowance TMR herd, were maintained indoors and consumed a mixed ration feed consisting of maize silage (40 %), concentrates (40 %) and grass silage (20 %) and the PMR herd rotated between outdoor rotational grazing following the morning milking and indoor mixed ration feeding after evening milking. The full diet composition are outlined in Fitzpatrick et al. (2022) and Timlin et al. (2023). Bulk milk consisted of a pooled morning and evening milking which were then aliquoted. Samples for fat-soluble compound composition were stored frozen until their analysis. Cows within each herd were balanced for pre-experiment daily milk yield and daily milk solids yield, economic breeding index, average calving date and lactation number. A total of 102 ($n = 34$) samples were collected and analysed for their fat-soluble compound composition as outlined below.

### 2.3. Extraction procedure

Samples were extracted using a modification of the procedure described by Stout et al. (2018). The samples were thawed and vortexed briefly before aliquoting 2 mL of milk to a 10 mL Pyrex glass test tube. The milk samples were saponified by the addition of 2 mL of ethanolic ascorbic acid (2 % w/v), and 3 mL of aqueous KOH (50 % w/v). An aliquot (200 μL) of internal standard (trans- β-apo-8′-carotenal) was added and the tubes were vortexed briefly and incubated in a water bath at 60 °C for 30 min. The tubes were then cooled in an ice bath. Water (HPLC grade, 1 ml) was added and the unsaponifiable lipids were extracted with 3 ml hexane: ethyl acetate (70:30, 0.005 % BHT w/v). The contents of the tube were thoroughly mixed by inversion and centrifuged at 2500 rpm for 5 min. The organic phase containing carotenoids was removed and the extraction was repeated. The pooled extracts were evaporated to dryness using a vacuum evaporator (Genevac, BioPharma Group, Winchester, UK). The dried residue was reconstituted using 200 μL of methanol ethyl acetate (4:1) and transferred to a HPLC vial. The extracted samples were typically analysed on the same day as extraction.

### 2.4. HPLC analysis

Carotenoids, retinol and α-tocopherol were quantified in the same chromatographic run using a Shimadzu LC-20AD High Performance Liquid Chromatography system (Shimadzu Corporation, Kyoto, Japan). The system consisted of two LC-20ADXR pumps, SIL-30AC autosampler, CTO-20AC column oven and an SPD-M30A photodiode array (PDA) detector and RF-20AXS fluorescence detector connected in series. An Ascentis Express C30 column (10 cm × 2.1 mm, 2.7 μm, Merck, Darmstadt, Germany) maintained at a temperature of 18 °C was used. Analytes were separated by gradient elution based on a previously reported method (Taylor et al., 2006). Mobile phases consisted of methanol:water (97:3) (mobile phase A) and tert-butyl methyl ether (mobile phase B). The gradient was initially set to 5 % B for 7.6 min, increased to 38 % B in 0.2 min, and held for 0.4 min, then increased to 68 % B in 0.2 min, and held for 4.3 min before returning to 5 % B in 1 min and allowed to re-equilibrate for 5 min. The total run time for the analysis was 20 min. The flow rate was set at 0.3 mL/min, with an injection volume of 5 μL. A Model SPD-M30A photodiode array detector monitored retinol and carotenoids at 325 nm and 450 nm, respectively. α-Tocopherol was monitored using a Model RF-20AXS fluorescence detector with excitation and emission wavelengths of 293 nm and 326 nm, respectively.

Data acquisition, analysis, and processing were carried out using LabSolutions Lite software (version 5.54 SP5) (Shimadzu Corporation, Kyoto, Japan). Lutein, zeaxanthin, β-carotene, retinol, and α-tocopherol levels in the samples were determined from the respective standard curves after correcting for extraction efficiency, based on the recovery of

β-apo-8′-carotenal. The limits of quantitation were 2 μg/100 ml milk for α-tocopherol, 0.5 μg/100 ml milk for β-carotene, 0.3 μg/100 ml milk for retinol and 0.2 μg/100 ml milk for Lutein and zeaxanthin. The limits of detection were 1 μg/100 ml milk for α-tocopherol, 0.17 μg/100 ml milk for β-carotene, 0.12 μg/100 ml milk for retinol and 0.1 μg/100 ml milk lutein and zeaxanthin.

### 2.5. Statistical analysis

Statistical analysis was conducted using the lmerTests package in R x64 4.2.1 (R Core Team, 2020) as outlined in Timlin et al. (2024) with the following exceptions. Diet and month were considered fixed effects and the week number was considered a random effect. A second model analysing the influence of stage of lactation (SOL) and diet*SOL was also operated, with SOL and diet*SOL considered fixed effects and week number considered a random effect. Datasets that failed a Shapiro Wilks test for the residuals ($P < 0.05$) were transformed by lambda (β-carotene, lutein and zeaxanthin) and the transformed datasets used to calculate the P-values for diet, month, SOL, diet*month and diet*SOL. Values below $P = 0.05$ were considered significant.

Correlations were conducted using the 'PerformanceAnalytics' package in R x64 4.2.1. Correlations involving the functional attributes of milk were made with the data published by Timlin et al. (2023). As outlined in Timlin et al. (2023), due to a Covid-19 lockdown preventing techno-functional property analysis of fresh milk samples collected between weeks 5 and 15. Therefore carotenoid results from weeks 1–4 and 16–35 exclusively were used for correlations with the techno-functional attributes. These attributes included the colour (L*, a* and b*) values and heat coagulation time of milk. As all datasets correlated (with the exception of L*-value) were not normally distributed (Shapiro Wilks $P < 0.05$), a Spearman's correlation analysis was conducted to correlate the carotenoid concentration and techno-functional attributes. Correlation coefficients $< 0.5$ were considered weak correlations, 0.5–0.7 were considered moderate and coefficients $> 0.7$ were considered strong correlations.

Data underwent log transformation for normalization prior to receiver operating characteristic (ROC) curve, partial least squares discriminatory analysis (PLSDA) and hierarchical clustering heatmap analysis. These analyses were conducted using MetaboAnalyst 5.0 (Pang et al., 2021; Xia et al., 2013).

## 3. Results and discussion

### 3.1. Feeding system

α-Tocopherol was the fat-soluble compound quantified in highest concentrations and zeaxanthin in the lowest concentrations in milks (Table 1). The relative proportion of each fat-soluble compound quantified in this study is in line with that of previous research (Havemose et al., 2004; Hulshof et al., 2006). Amongst the fat-soluble compounds of forage, lutein has one of the highest relative abundances and α-tocopherol one of the lowest relative abundances (Chauveau Duriot et al., 2010; Rapisarda et al., 2023). However, among the fat-soluble-compounds in milk in this study, lutein had one of the lowest relative abundances and α-tocopherol the highest. A similar trend in milk was exhibited in previous research (Chauveau Duriot et al., 2010). The higher relative abundance of α-tocopherol in milk compared to forages may result from the low intestinal digestibility of natural α-tocopherol in feed (Lashkari et al., 2023), resulting in a large proportion of dietary α-tocopherol available for uptake into milk. The lower relative abundance of lutein in milk compared to forage suggests there is an increased metabolic breakdown of dietary lutein compared to other fat-soluble compounds, resulting in less lutein availability for uptake into milk. However, information on the metabolism of lutein in ruminants is currently verylimited. An analysis of the fat-soluble nutritional composition of the feeds used in each feeding system would have been beneficial in further understanding the metabolism of the various compounds and their uptake into milk, however, this was not possible in

**Table 1**
Concentration of zeaxanthin, α-tocopherol, β-carotene, retinol and lutein in milk (μg/100 mL) from herds consuming a high (GRS), medium (PMR) or no (TMR) pasture allowance across lactation.

| | | Early Lactation | | | Mid Lactation | | | Late Lactation | | | p-value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Compound | Diet | March | April | May | June | July | August | September | October | Average | Diet | Month | Diet*Month |
| Zeaxanthin μg/100 mL | GRS | 1.34 ±0.41 | 0.75 ±0.16 | 1.51 ±0.71 | 0.75 ±0.12 | 1.02 ±0.38 | 1.13 ±0.23 | 1.02±0.19 | 0.79 ±0.14 | 1.03 ±0.40[a] | <0.001 | 0.017 | 0.074 |
| | PMR | 0.92 ±0.33 | 0.51 ±0.20 | 0.81 ±0.41 | 0.25 ±0.09 | 0.36 ±0.11 | 0.44 ±0.11 | 0.42±0.09 | 0.39 ±0.09 | 0.50 ±0.29[b] | | | |
| | TMR | 1.00 ±0.37 | 0.51 ±0.19 | 0.37 ±0.12 | 0.27 ±0.03 | 0.46 ±0.15 | 0.41 ±0.08 | 0.33±0.05 | 0.37 ±0.10 | 0.45 ±0.26[b] | | | |
| α-Tocopherol μg/100 mL | GRS | 140.79 ±27.78 | 140.72 ±26.68 | 154.77 ±41.74 | 118.98 ±9.93 | 169.57 ±50.10 | 173.47 ±10.83 | 171.29 ±25.69 | 160.57 ±25.83 | 153.26 ±32.23[a] | <0.001 | 0.008 | 0.005 |
| | PMR | 100.08 ±15.32 | 91.78 ±23.61 | 92.11 ±21.99 | 61.58 ±14.96 | 88.04 ±17.16 | 93.17 ±11.84 | 97.79 ±7.60 | 82.43 ±11.25 | 87.86 ±18.70[b] | | | |
| | TMR | 86.84 ±16.32 | 66.90 ±15.89 | 72.82 ±21.43 | 65.90 ±6.28 | 107.13 ±26.36 | 95.23 ±9.16 | 86.57 ±13.60 | 63.94 ±9.09 | 80.40 ±20.30[b] | | | |
| β-carotene μg/100 mL | GRS | 3.62 ±0.44 | 2.99 ±0.18 | 6.44 ±2.36 | 4.52 ±1.29 | 5.84 ±1.49 | 4.72 ±1.28 | 3.75±0.65 | 3.05 ±0.78 | 4.35 ±1.60[a] | <0.001 | 0.049 | 0.095 |
| | PMR | 3.14 ±0.48 | 2.61 ±0.42 | 3.82 ±1.65 | 3.22 ±1.71 | 3.34 ±0.71 | 2.54 ±0.32 | 2.96±0.52 | 1.93 ±0.34 | 2.95 ±1.01[b] | | | |
| | TMR | 2.97 ±0.21 | 2.01 ±0.19 | 3.00 ±1.86 | 2.99 ±1.18 | 3.11 ±0.73 | 2.27 ±0.45 | 2.46±0.51 | 1.43 ±0.17 | 2.54 ±0.95[c] | | | |
| Retinol μg/100 mL | GRS | 14.06 ±3.15 | 13.81 ±6.57 | 13.57 ±0.90 | 10.69 ±2.31 | 16.40 ±6.93 | 11.60 ±0.64 | 17.91 ±4.27 | 17.01 ±1.76 | 14.38 ±4.34[a] | <0.001 | 0.034 | 0.023 |
| | PMR | 11.22 ±2.88 | 11.55 ±3.28 | 8.56 ±1.99 | 6.11 ±2.28 | 10.46 ±4.01 | 8.19 ±1.94 | 10.35 ±0.74 | 10.26 ±1.16 | 9.51 ±2.82[b] | | | |
| | TMR | 8.70 ±1.56 | 10.26 ±4.91 | 6.82 ±1.38 | 7.30 ±2.21 | 11.48 ±3.21 | 5.74 ±0.95 | 8.36±1.13 | 8.03 ±1.57 | 8.31 ±2.74[b] | | | |
| Lutein μg/100 mL | GRS | 5.16 ±0.92 | 3.79 ±1.07 | 5.49 ±2.45 | 3.42 ±0.51 | 4.84 ±1.41 | 5.32 ±0.71 | 5.53±0.50 | 5.07 ±1.06 | 4.81 ±1.31[a] | <0.001 | 0.001 | <0.001 |
| | PMR | 3.49 ±1.53 | 2.15 ±0.89 | 2.94 ±1.68 | 1.12 ±0.37 | 1.69 ±0.48 | 2.66 ±0.16 | 2.78±0.36 | 2.18 ±0.40 | 2.35 ±1.07[b] | | | |
| | TMR | 1.84 ±0.67 | 1.08 ±0.36 | 1.26 ±0.44 | 0.99 ±0.13 | 1.85 ±0.56 | 1.43 ±0.33 | 1.22±0.12 | 1.16 ±0.37 | 1.34 ±0.47[c] | | | |

the present study due to limitations.

The level of pasture feeding significantly altered the concentrations of all fat-soluble compounds quantified ($P < 0.001$). Most notably, the GRS feeding system had a significantly higher proportion of each compound (β-carotene, retinol, lutein, zeaxanthin and α-tocopherol) than both TMR and PMR milks ($P < 0.001$). This is illustrated by the clustering heatmap in Fig. 1 whereby the heatmap sub clusters PMR and TMR milks together as a result of the relative abundance of each fat-soluble compound. Similar differences in the fat-soluble compound concentration of milk as a result of feeding system were noted in milks derived from grass silage vs maize silage (Havemose et al., 2004), from low input farms compared to high input farms (Butler et al., 2008) and in milks from cows fed hay compared to corn silage (Graulet et al., 2019).

Concentrations of both β-carotene ($P = 0.001$) and lutein ($P < 0.001$) were intermediate in PMR milks and lowest in TMR milks. Concentrations of retinol, zeaxanthin and α-tocopherol were also intermediate in PMR and lower in TMR, however, concentrations were not significantly different between the milks of PMR and TMR ($P > 0.05$). The is illustrated in the PLSDA (Fig. 2), whereby GRS and TMR samples can clearly be distinguished as a result of their two principal components, however, PMR clearly overlaps both GRS and TMR. Concentrations of retinol, β-carotene, and α-tocopherol in milk have previously been reported to depend primarily on the dietary intakes of these compounds by the animal through their feed and, to a lesser extent, the difference in fat content of the resulting milk produced (Graulet, 2014). Despite cereals being amongst the most used animal feeds in dairy production systems around the world, cereals such as maize only contain low amounts of carotenoids (Meléndez-Martínez et al., 2022; Zhai et al., 2016). Additionally, concentrates typically contain very low concentrations of carotenoids (Nozière et al., 2006). Therefore, considering that maize and concentrates contain low concentrations of carotenoids compared to pastures (Messias et al., 2014; Rapisarda et al., 2023), maintain a substantial proportion of the feed used in TMR and PMR systems, and since carotenoids cannot be produced de novo (Arunkumar et al., 2020; Metibemu & Ogungbe, 2022), it is not surprising to see the carotenoid concentration of milks derived from PMR and TMR systems contain significantly ($P < 0.05$) lower concentrations of carotenoids than GRS. Furthermore, by ensiling pasture forages into grass silage, the carotenoid concentration of the feed can be reduced by 83 % compared to the fresh pasture alternative, primarily resulting from solar radiation (Chauveau-Duriot et al., 2005; Nozière et al., 2006). Therefore the use of ensiled grass as opposed to fresh grass in the mixed ration feed in this study (20 % on DM basis) will also have contributed to the lower dietary intake of carotenoids in TMR and PMR systems and consequently in the resulting milks of TMR and PMR compared to GRS.

The fat-soluble compound concentrations in milk were tested in an ROC curve model to assess their use as biomarkers of pasture feeding. More information on the use of area under the curve (AUC) tests for biomarker analysis are discussed in Timlin et al. (2023) and Timlin et al. (2024). When used individually, three compounds were considered excellent (AUC > 0.90) biomarkers at differentiating milks from GRS and TMR systems (lutein, α-tocopherol and zeaxanthin), two considered excellent at differentiating milks from GRS and PMR systems (α-tocopherol and lutein) and lutein was considered very good (AUC = 0.8 – 0.9) at differentiating milks from TMR and PMR systems (Table 2). All five compounds were inputted into a new ROC model more capable of accounting for biological variation (Naviaux et al., 2016). This model showed an excellent (> 0.90) ability to differentiate between milks derived from GRS and TMR systems, as illustrated by the AUC score of 0.995 (with a confidence interval (95 % CI) = 0.975 – 1), an excellent ability of distinguishing between milks derived from GRS and PMR systems (AUC = 0.951, 95 % CI = 0.839 – 1) and an excellent ability of distinguishing between milks derived from TMR and PMR systems (AUC = 0.953, 95 % CI = 0.859 – 1). Therefore, this model highlights an excellent capability of distinguishing milks derived from high, medium and no pasture allowance feeding systems. To the authors' best knowledge, this appears to be the first time that fat-soluble compound concentrations of milk were assessed for their use as biomarkers of pasture feeding. Despite these strong results, the model is slightly less efficient than the model developed using fatty acid proportions and b* colour values on the same milk samples (Timlin et al., 2023). Similarly, several other authors have highlighted the potential of milk fatty acids for authentication of pasture derived products.

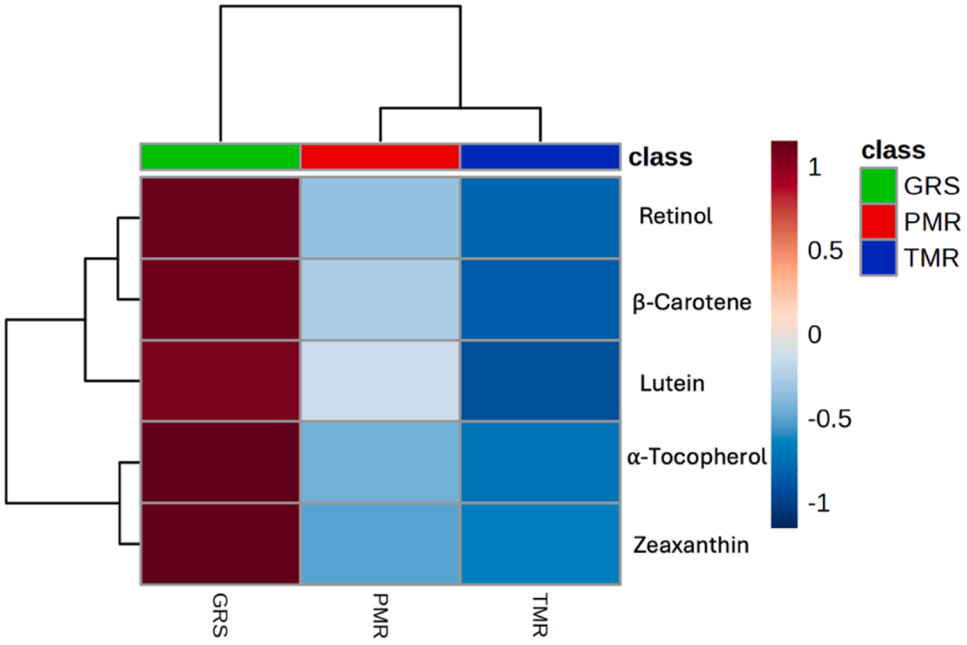



**Fig. 1.** Hierarchal clustering heatmap of fat-soluble compounds derived in milk (μg/mL) from herds consuming a high (GRS), medium (PMR) or no (TMR) pasture allowance.

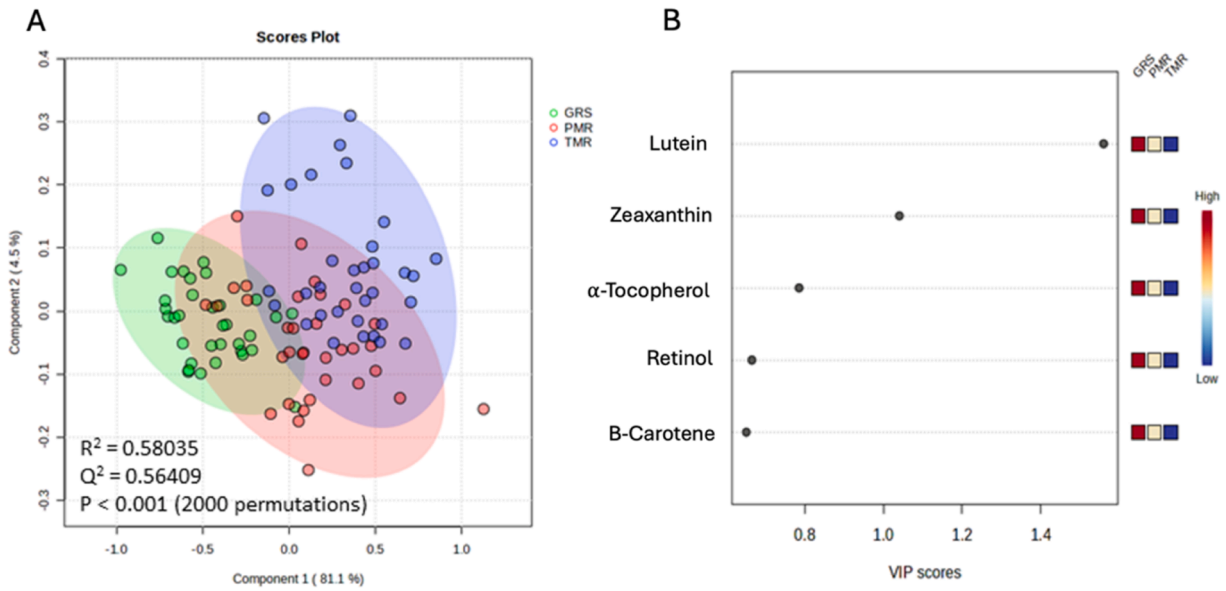



**Fig. 2.** Partial least squares discriminatory analysis (A) and variable in projection scores (B) of milks derived from herds consuming high (GRS), medium (PMR) or no (TMR) pasture allowance.

**Table 2**
Area under the curve scores using individual fat-soluble compound concentrations in milk (μg/mL) as biomarkers of pasture feeding between high and low (GRS-TMR), high and medium (GRS-PMR) and medium and no (PMR-TMR) pasture allowances.

| | Area under the curve | | |
|---|---|---|---|
| | GRS-TMR | GRS-PMR | PMR-TMR |
| β-carotene | 0.85 | 0.78 | 0.63 |
| Retinol | 0.88 | 0.83 | 0.65 |
| Lutein | 1.00 | 0.93 | 0.82 |
| α-tocopherol | 0.97 | 0.97 | 0.63 |
| Zeaxanthin | 0.92 | 0.89 | 0.56 |

### 3.2. Stage of lactation

All carotenoids (with the exception of retinol which is a breakdown product of β-carotene and not found in animal feed) had an observed increase in concentration in GRS milks in May, followed by a decrease in June, which was not necessarily evident in TMR milks (Figs. 3–7). As this only occurs in the diets of herds consuming high or medium proportions of pasture, the observed increase in carotenoid concentrations is not necessarily as a result of the physiological state of the animal, but rather due to seasonal pasture growth patterns. Grass growth patterns across Ireland for the months of May – July (O'Donovan et al., 2022) show a similar trend as that of carotenoid concentration in milks over the same time period and therefore may have influenced the carotenoid concentration in pasture and consequently the concentration of carotenoids in milk. Additionally, the use of perennial ryegrass swards as the primary pasture source in Ireland may have resulted in a reduced digestibility of grass pastures in June, brought about by the transition of perennial ryegrass to a reproductive stage (Tubritt, 2021). As a result of this lower digestibility, the pastures may have a lower carotenoid concentration available for metabolism and uptake into milk. Further investigation would be required to confirm this theory as there appears to be no research at present outlining the influence of plant maturity stage on the carotenoid concentration of perennial ryegrass pastures. A decrease in the concentration of carotene and vitamin A has however been reported in the milk of cows consuming a mature ryegrass (*Lolium hybrid* $H_1$) compared to that of cows consuming immature ryegrass (McDowall & McGillivray, 1963). Similarly, a slight decrease in carotenoid concentration has been reported in mountain grassland pastures with increasing maturity stage (Calderón et al., 2006). A similar decrease in the concentration of fat-soluble compounds is therefore likely in perennial ryegrass pastures with increasing maturity due to the decreasing leaf to stem ratio during the reproductive period and the association between the plant leaf to stem ratio and antioxidant concentration (Ballet et al., 2000; Marino, 2012).

The reason for the higher concentration of zeaxanthin in early lactation milk is difficult to find as previous research has struggled to quantify zeaxanthin in milk (Calderón et al., 2007, 2006; Chauveau Duriot et al., 2010), despite their quantification in plasma (Calderón et al., 2007; Chauveau Duriot et al., 2010) and feed (Calderón et al., 2006; Chauveau Duriot et al., 2010). The higher concentration of zeaxanthin quantified in milks from all systems in March, and milks from GRS and PMR systems in May, is evidently responsible for the significantly higher concentration of zeaxanthin in early lactation compared to both mid ($P = 0.005$) and late ($P = 0.031$) lactation. The decreasing concentration of zeaxanthin in all diets between March and April would suggest that there was an increasing energy balance experienced by cows over this period (Butler, 2005; Timlin et al., 2021) which had a significant effect on the metabolism of zeaxanthin and therefore reduced the concentration of zeaxanthin uptake into milk in April. The spike in zeaxanthin concentration in May from both high and medium pasture allowance systems (GRS and PMR) would suggest that this spike was influenced by an increased zeaxanthin concentration in pasture during May as discussed in the previous paragraph.

### 3.3. Correlations with milk colour

Values for techno-functional properties including L*, a* and b* values were obtained from the study of (Timlin et al., 2023). Strong correlations were made between the b* colour of milk with the concentration of both lutein (0.81) and zeaxanthin (0.77), and a moderate correlation with retinol (0.63) and α-tocopherol (0.69) (Fig. 8). Lutein and zeaxanthin both provide a yellow or orange colour to their host (Kalariya et al., 2012), therefore their strong positive correlations with the yellow colour of milk in this study is not surprising. The weak correlation coefficient between β-carotene and b*-value is however surprising, given the strong correlations exhibited in dairy products previously (O'Callaghan et al., 2016, 2017). This may partly be as a result of the different dairy product being examined in this study.

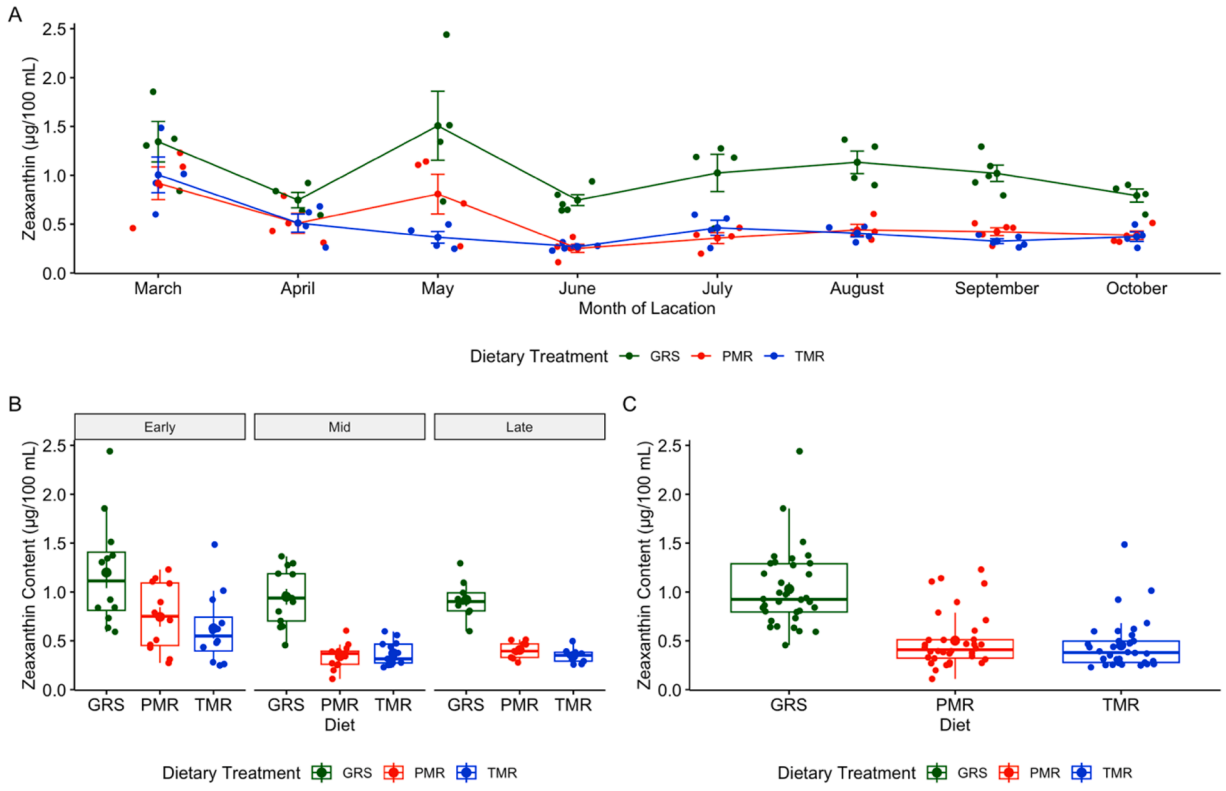



**Fig. 3.** Concentration of zeaxanthin in milk (µg/mL) derived from herds consuming high (GRS), medium (PMR) or no (TMR) pasture allowances across lactation (A), stage of lactation (B) and dietary average (C).

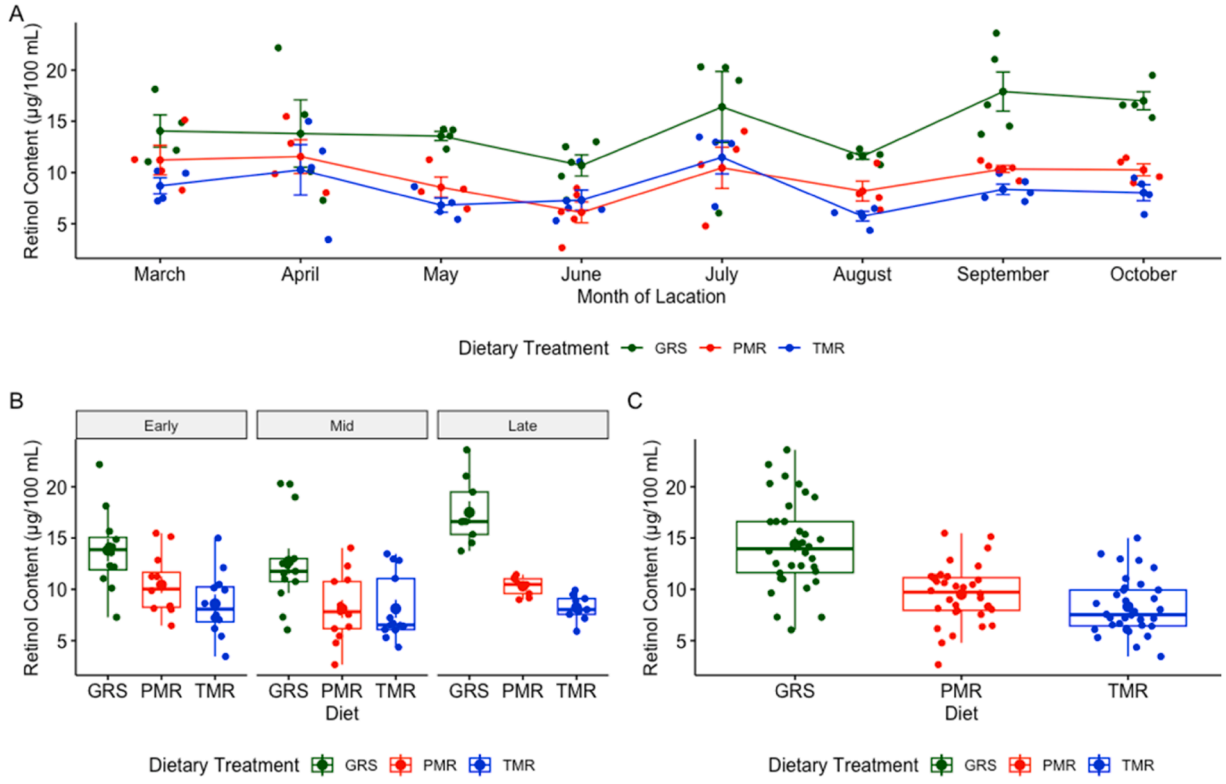



**Fig. 4.** Concentration of retinol in milk (µg/mL) derived from herds consuming high (GRS), medium (PMR) or no (TMR) pasture allowances across lactation (A), stage of lactation (B) and dietary average (C).

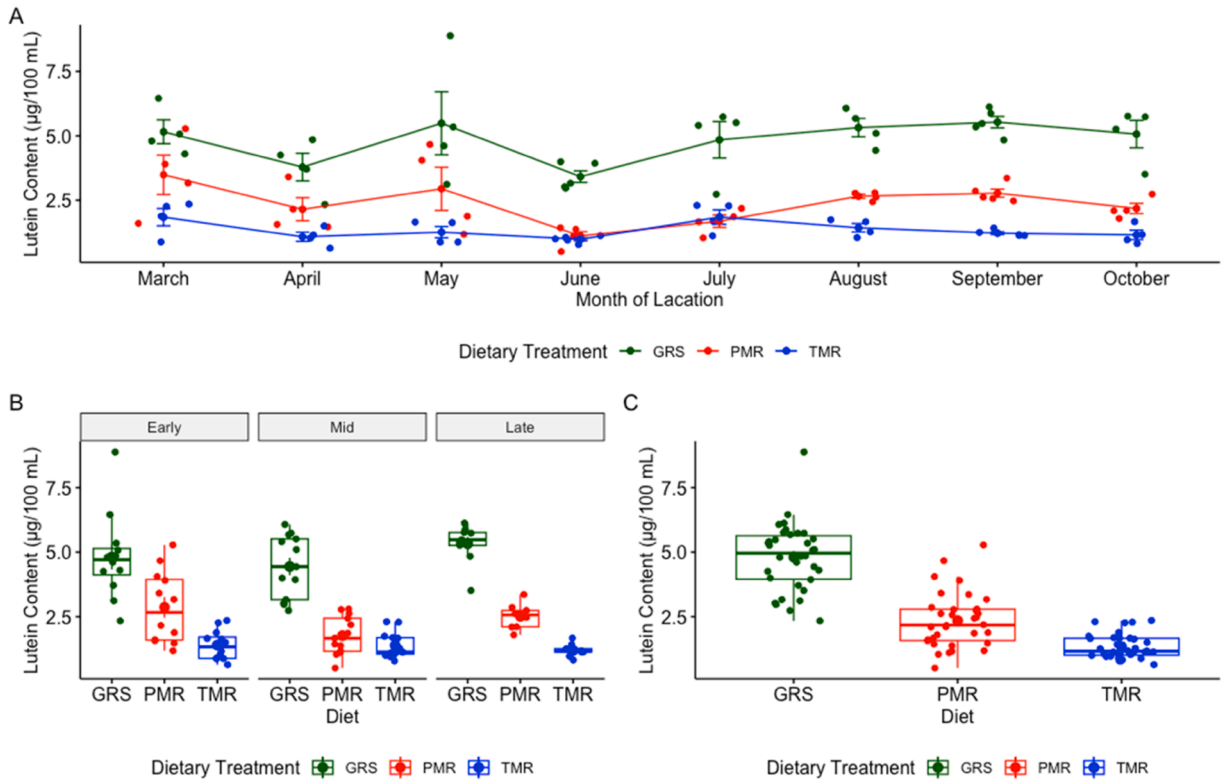



**Fig. 5.** Concentration of lutein in milk (µg/mL) derived from herds consuming high (GRS), medium (PMR) or no (TMR) pasture allowances across lactation (A), stage of lactation (B) and dietary average (C).

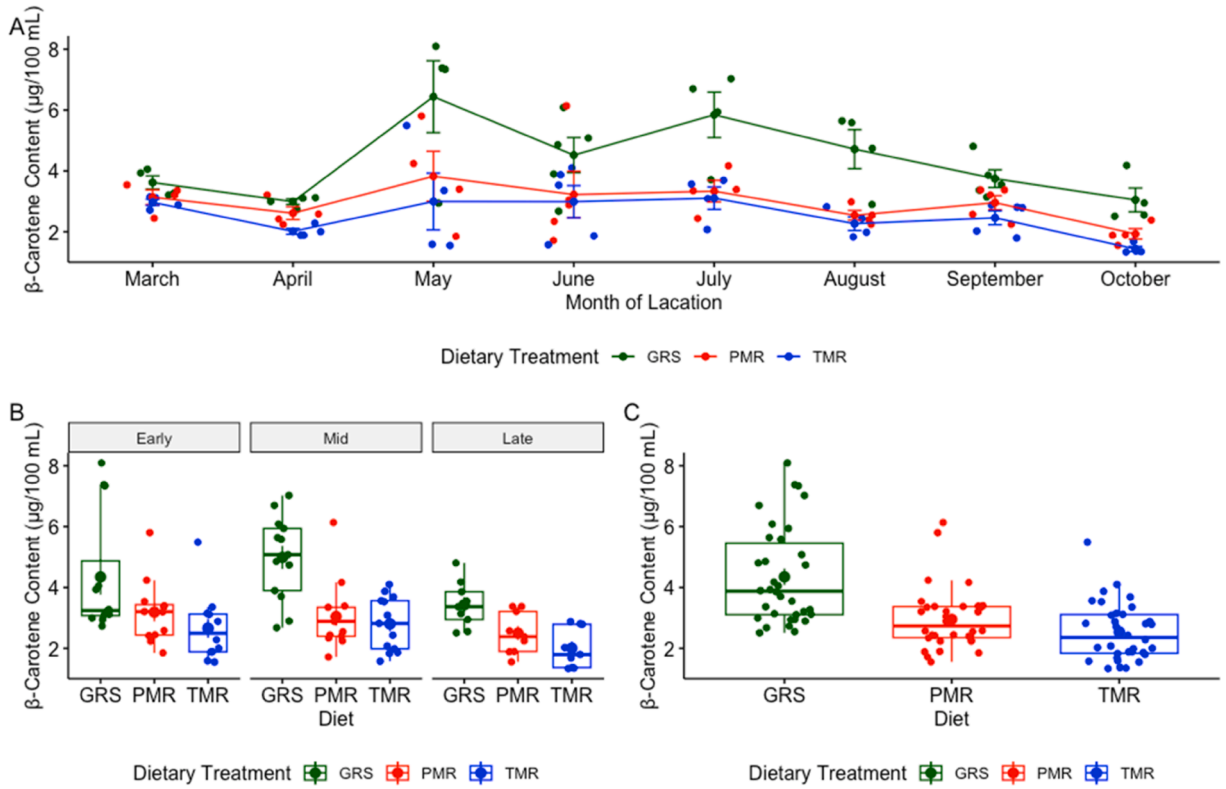



**Fig. 6.** Concentration of β-carotene in milk (µg/mL) derived from herds consuming high (GRS), medium (PMR) or no (TMR) pasture allowances across lactation (A), stage of lactation (B) and dietary average (C).

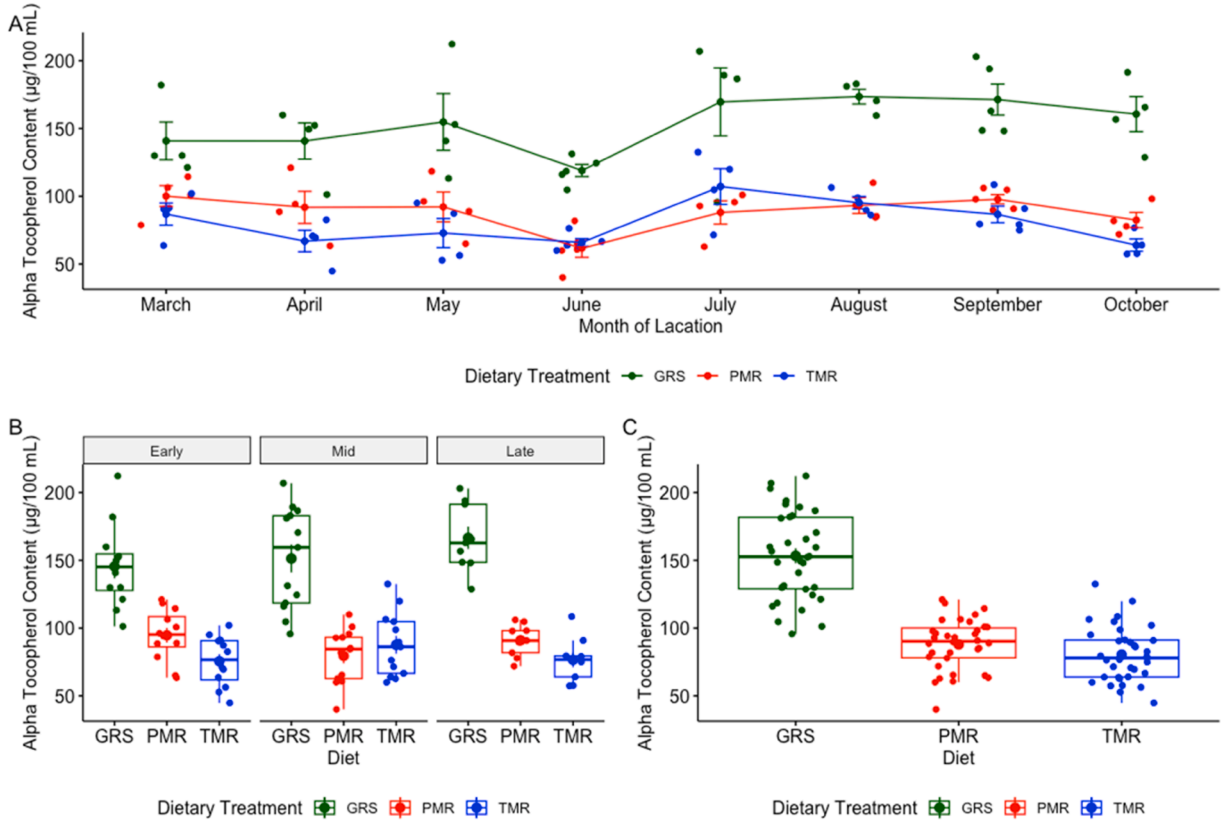



**Fig. 7.** Concentration of α-tocopherol in milk (µg/mL) derived from herds consuming high (GRS), medium (PMR) or no (TMR) pasture allowances across lactation (A), stage of lactation (B) and dietary average (C).

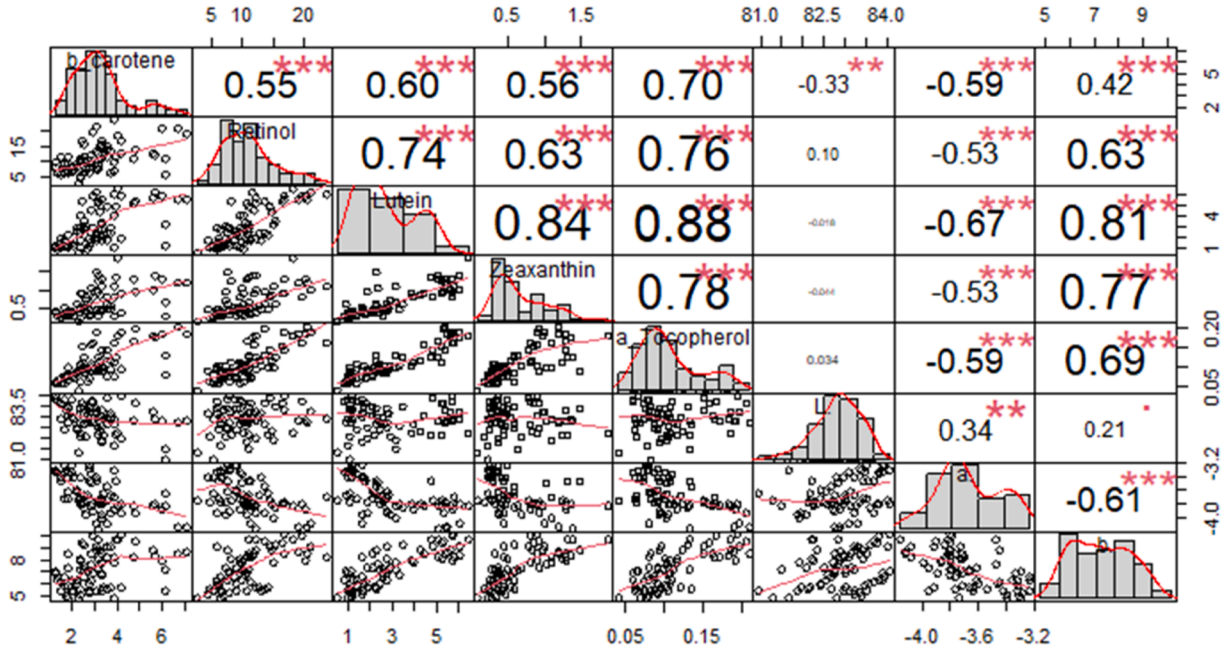



**Fig. 8.** Spearmans correlation between fat-soluble compounds in milk and their respective colour values (L*, a* and b*).

### 3.4. Study limitations

This study was focused on a targeted selection of relatively abundant fat-soluble compounds, however, an untargeted or semi targeted lipidomics or metabolomics approach using Ultra High-Performance Liquid chromatography-Mass Spectrometry (UHPLC-MS) platform or Nuclear Magnetic Resonance (NMR) could have potentially identified other novel compounds, possibly present in trace quantities, with biomarker potential. Rocchetti and O'Callaghan (2021) have recently highlighted the potential for metabolomics in its applications for milk

quality and traceability. Another challenge lies in the extrapolation of the findings of this research to the finished dairy products. Various processing techniques involved in the preparation of dairy products could impact the concentration of fat-soluble compounds, hence the influence of such confounding parameters warrants further investigation.

## 4. Conclusion

The results from this study outline how increasing the levels of pasture in the animal feeding system increases the concentration of fat-soluble compounds in milk including β-carotene, retinol, lutein, zeaxanthin and α-tocopherol. Similarly, the impact of seasonal grass pasture growth and stage of lactation also evidently impacted the fat-soluble compound concentration of milk. Such differences may have a significant impact on the consumer acceptability of such products as a result of the altered milk colour. The use of fat-soluble compounds examined in this study as biomarkers were very successful and if coupled with previously outlined fatty acid models, have the potential to offer a greater robustness for authentication applications. This study highlights the potential of differentiating milks from pasture and non-pasture-fed systems based on each carotenoid proportion.

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## CRediT authorship contribution statement

**Mark Timlin:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Janhavi Sanjay Dhumal:** Investigation, Formal analysis, Data curation. **Raghunath Pariyani:** Writing – review & editing, Formal analysis, Data curation. **Karen Galvin:** Writing – review & editing, Methodology, Formal analysis, Data curation. **John P. Murphy:** Writing – review & editing, Methodology, Investigation. **Michael O'Donovan:** Writing – review & editing, Methodology, Investigation. **Deirdre Hennessy:** Writing – review & editing, Methodology, Investigation. **André Brodkorb:** Writing – review & editing, Investigation. **Alice J. Lucey:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Tom F. O'Callaghan:** Writing – review & editing, Project administration, Investigation, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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