Effect of phytol in forage on phytanic acid content in cow’s milk

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Objective: Bioactive compounds in ruminant products are related to functional compounds in their diets. Therefore, this study aimed to explore the effect of forage sources, Italian ryegrass (IR) silage vs corn silage (CS) in the total mixed ration (TMR), on milk production, milk composition, and phytanic acid content in milk, as well as on the extent of conversion of dietary phytol to milk phytanic acid.

Methods: Phytanic acid content in milk was investigated for cows fed a TMR containing either IR silage or CS using 17 cows over three periods of 21 days each. In periods 1 and 3, cows were fed CS-based TMR (30% CS), while in period 2, cows were fed IR silage-based TMR (20% IR silage and 10% CS).

Results: The results showed that there were no differences in fat, protein, lactose, solids-not-fat, somatic cell count, and fatty acid composition of milk among the three experimental periods. There were no differences in the plasma concentration of glucose, triglycerides, total cholesterol, and nonesterified fatty acids among the three experimental periods, while the blood urea nitrogen was higher (p<0.05) in period 2. The milk phytanic acid content was higher (p<0.05) in period 2 (13.9 mg/kg) compared with periods 1 (9.30 mg/kg) and 3 (8.80 mg/kg). Also, the phytanic acid content in the feces was higher (p<0.05) in period 2 (1.65 mg/kg dry matter [DM]) compared with period 1 (1.15 mg/kg DM), and 3 (1.17 mg/kg DM). Although the phytol contents in feces did not differ among the three feeding periods, the conversion ratio from dietary phytol to milk phytanic acid was estimated to be only 2.6%.

Conclusion: Phytanic acid content in cow’s milk increases with increasing phytol content in diets. However, phytol might not be completely metabolized in the rumen and phytanic acid, in turn, might not be completely recovered into cow’s milk. The change of phytanic acid content in milk may be positively correlated with the change of phytol in the diet within a short time.

Keywords: Milk; Phytanic Acid; Phytol; Silage

INTRODUCTION

Ruminant products (milk and meat) are known to contain bioactive compounds that contribute to human health [1]. Among these compounds which have beneficial properties for human health that contribute to metabolic syndrome prevention, include phytanic acid (a fatty acid) derived from the phytol moiety of chlorophyll [2,3]. The phytanic acid content in milk has been reported to vary with forages level, species, and conservation methods depending on the phytol content of feed; it increases with increasing fresh forage [4,5], silage instead of hay [6], or red clover rather than grass silage [7] intake. The determinant factors of phytanic acid content in ruminant products are the chlorophyll content in forage and the amount of phytol liberated in the rumen. Therefore, effective utilization of chlorophyll and phytol in forage could improve the additional value of ruminant products and could have positive effects on the health of cows. Despite some papers reported the phytanic acid content in milk products [4] and the change of chlorophyll and phytol in
herbage during the preservative process [8,9], no reports confirmed the relationship between the phytol intake and phytic acid in milk.

Since more attention being paid to organic farming, making flexible use of forage is an important strategy for agricultural development in the future, and exploring the potential value of forage is a necessary study. Italian ryegrass (IR; *Lolium multiflorum* Lam.), is one of the most important forage crops. IR is now widely distributed through temperate areas of the world and generally regarded as the basis of grassland improvement because of its high nutritional value, digestibility, and well ensiling characteristics [10]. IR is also used as a major silage crop in Japan and has been widely used for silage making [11]. Whole crop corn silage (CS) contains leaves, steams, grains and cobs so that chlorophyll or phytol content would be diluted with the non-leaf part of the plant. Thus, the phytol content is expected to be higher in IR silage compared with CS. In dairy production systems, total mixed rations (TMR) containing forages, grains, protein feeds, minerals, vitamins, and feed additives are used to satisfy the nutrient requirement of cows [12]. The phytanic acid content in milk of cows fed TMR containing IR silage is expected to be higher than that of cows fed TMR containing CS, due to the difference in the phytol content between IR silage and CS.

Therefore, this study aimed to explore the effect of forage sources, IR silage vs CS in the TMR on milk production, milk composition, and phytanic acid content in milk, and the extent of conversion of dietary phytol to milk phytanic acid.

**MATERIALS AND METHODS**

**Experimental design and animals**

All animal procedures were managed according to the guidelines of the Animal Care and Use Committee of Hiroshima University. A total of 17 Holstein cows (8 primiparous and 9 multiparous cows) averaging (mean±standard deviation) 1.9±1.2 parity, 213±97 days in milk, 732±65 kg of body weight, and 31.3±8.8 kg/d milk production, were used in the experiment consisting of three 21-d periods at Hiroshima University Farm. Cows were raised in the cowshed installing an automatic milking system (Astronaut A3 next, Lely, the Netherland) and the roughage intake control system (Insentec, Drachten, the Netherland). Cows were supplied with a concentrate diet with an automatic feeder in the automatic milking system. The CS-based TMR was fed during the first and third periods (period 1 and period 3), and the IR silage-based TMR was fed during the second period (period 2). The ingredients and chemical composition of the TMR are shown in Table 1. The milk samples were collected at the last 2 days of each period and preserved at −30°C for the later determination of the milk components. The blood of the caudal artery was collected at 13:00 on the last day of each period. Then, plasma was collected after centrifugation at 4°C for 10 min and preserved at −30°C for further analyses. Feed samples were collected over the last 3 days of each experimental period and freeze-dried for later analysis. Spot fecal samples were collected

| Item                  | Period -1 | Period -2 | Period -3 | SEM       |
|-----------------------|-----------|-----------|-----------|-----------|
| **Ingredient (% of DM)** |           |           |           |           |
| Italian ryegrass silage | 0         | 20.4      | 0         | -         |
| Corn silage            | 30.2      | 9.5       | 30.2      | -         |
| Oats hay               | 9.9       | 7.1       | 9.9       | -         |
| Alfalfa hay            | 11.1      | 12.7      | 11.1      | -         |
| Beet pulp              | 6.6       | 6.9       | 6.6       | -         |
| Concentrate mixture    | 40.2      | 41        | 40.2      | -         |
| CaCO₃                  | 0.8       | 1         | 0.8       | -         |
| Vitamin                | 0.9       | 1         | 0.9       | -         |
| NaCl                   | 0.4       | 0.4       | 0.4       | -         |
| **Composition (% of DM)** |           |           |           |           |
| Dry matter (% of FM)   | 44.7±0.96 | 46.0±2.20 | 47.1±0.43 | 5.96      |
| Crude protein          | 12.6±0.21 | 13.0±0.40 | 13.5±0.59 | 0.56      |
| NDFom                  | 40.3±0.09 | 42.3±0.93 | 41.0±0.89 | 1.66      |
| Ether extract          | 3.15±0.09 | 3.09±0.06 | 3.1±0.11  | 0.03      |
| Ca                     | 0.85      | 0.83      | 0.8       | -         |
| P                      | 0.38      | 0.37      | 0.36      | -         |
| TDN                    | 66.9      | 68.7      | 69.3      | -         |
| Phytol (g/kg DM)       | 0.483±0.01 | 0.784±0.08 | 0.517±0.02 | 0.007    |

SEM, standard error of means; DM, dry matter; FM, fresh matter; NDFom, neutral detergent fiber exclusive of residual ash; TDN, total digestible nutrients.

1) Cows were fed corn silage TMR (periods 1 and 3) and Italian ryegrass silage TMR (period 2).

b Means with different letters significantly differ (p<0.05).
from 4 cows over the last 3 days of each experimental period. The feces of each cow were collected immediately after defecation in the morning (8:30 to 9:30), afternoon (16:30 to 17:30), and evening (00:30 to 1:30), then they were mixed completely and freeze-dried for later analysis.

**Chemical analysis**

Feed samples were analyzed for dry matter (DM), crude ash, crude protein (CP), and ether extract by the methods of AOAC [13], and the neutral detergent fiber (NDF) was determined according to Van Soest et al [14].

Milk samples were measured for fat, protein, lactose, and solids-not-fat (SNF) by an infrared analyzer (Lactoscope Filter C4+, Delta Instruments, Drachten, and the Netherlands). Somatic cell count (SCC) was analyzed by milk somatic cell counter (NucleoCounter, SCC-100, chemometec. Allerod, Denmark). Plasma samples were analyzed for glucose, non-esterified fatty acid (NEFA), triglyceride (TG), total cholesterol (T-CHO), and blood urea nitrogen (BUN) using an automated biochemical analyzer (AU 480; Beckman Coulter, Brea, CA, USA).

The fatty acid content including phytic acid in milk samples was determined by gas chromatography/mass spectrometry (GC-MS) (QP2010, Ultra, Shimadzu, Kyoto, Japan) after acid methylation of lipid extracts according to the methyl esterification method for fatty acid analyses of feeds [15]. Briefly, 1 mL of milk was mixed with 0.2 mL 28% ammonia solution and 0.8 mL 96% ethanol. Then, 0.2 mL of methyl tridecanoate (2.5 mg/mL hexane) was added as an internal standard. 1 mL 0.025% butylated hydroxytoluene diethyl ether and one mL hexane were added and mixed well. The supernatant after centrifugation at 1,710 g for 5 min was collected. The extraction process was repeated and the combined supernatant was dried under N₂ gas stream. Then, 3 mL 0.78 N hydrochloric acid (HCl) in methyl alcohol and 2 mL chloroform were added to the tube, and the tube was heated for 2.5 h at 65°C. Then 5 mL solution of 6% K₂CO₃ and 3 mL hexane was mixed completely. After centrifugation, the supernatant was loaded onto a column containing 0.5 g florisil, and then the column was eluated with 5 mL hexane with diethyl ether (95:5 V/V). The eluate was dried at 40°C under a constant stream of N₂ gas and redissolved in 1 mL hexane for gas chromatography-mass spectrometer (QP2010, Ultra, Shimadzu, Japan) equipped with SP-2560 (100 m×0.25 mm, film thickness 0.2 μm; Supelco, Bellefonte, PA, USA). Helium was used as a carrier gas. The column pressure was set at 170 kPa. The initial temperature of the column oven at 70°C for 3 min was raised to 130°C by 11°C/min, then to 160°C by 1°C/min, finally raised to 220°C by 3°C/min. The split ratio was 60.0. The injection volume was 1 μL. For the mass spectrometer, ion source temperature and interface temperature were set at 200°C and 240°C respectively. Selected ion mode was used to measure the relative intensity of 101 and 87 m/z fragments as target ions of methyl ester of phytic acid and nonadecanoic acid, respectively. Phytol in TMR and feces was analyzed following the methods of Liljenberg and Odham [16] and Takeda et al [17]; detailed analytical procedures are previously described [8]. Phytanic acid in feces was also determined by GC-MS (QP2010, Ultra, Shimadzu, Japan). Tridecanoic acid (0.25 mg/mL, 1 mL) was used as an internal standard solution. In a screw-capped tube, the internal standard solution (1 mL) was added to 0.1 g freeze-dried feces samples and then dried under N₂ stream at 40°C. And the following treatments were the same as the above methods.

**Statistical analysis**

Statistical analysis was performed using the general linear model procedure of SAS [18]. The data were analyzed as a complete blocked design. Tukey’s test was used to identify the differences of means (p<0.05) among experimental periods.

**RESULTS**

Chemical compositions of the TMR had no differences in DM, CP, and NDF among the periods (Table 1). The phytol content in the TMR at period 2 (0.784 g/kg DM) was higher (p<0.05) than that at periods 1 (0.483 g/kg DM) and 3 (0.517 g/kg DM). The DMI of TMR and concentrate were 19.5, 19.7, 20.1 kg/d and 4.9, 4.8, 4.6 kg/d, for periods 1, 2, and 3, respectively, but there were no significant differences among the three experimental periods (Table 2). The daily milk yield averaged 28.4 kg/d which was similar among the three experimental periods. There were no differences in fat, protein, lactose, SNF, and SCC content in milk among the three experimental periods (Table 2). There were no differences in fatty acid composition in milk among the three experimental

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**Table 2. Effects of feeding periods on feed intake, milk yield, and milk composition in dairy cows**

| Item            | Period -1 | Period -2 | Period -3 | SEM  |
|-----------------|-----------|-----------|-----------|------|
| DMI (kg/d)      | 19.5±0.56 | 19.7±3.72 | 20.1±0.51 | 2.33 |
| TMR             | 4.88±0.15 | 4.75±0.13 | 4.62±0.11 | 0.26 |
| Concentrate     |           |           |           |      |
| Milk yield (kg/d) | 28.8±1.36 | 29.2±1.40 | 27.3±1.57 | 1.45 |
| Milk composition (%) |       |           |           |      |
| Fat             | 4.03±0.08 | 4.14±0.09 | 4.09±0.07 | 0.34 |
| Protein         | 3.48±0.05 | 3.45±0.05 | 3.56±0.05 | 0.136|
| Lactose         | 4.57±0.03 | 4.57±0.04 | 4.54±0.04 | 0.076|
| SNF             | 8.97±0.06 | 9.03±0.05 | 9.02±0.06 | 0.183|
| SCC (×1,000/mL) | 138±45    | 145±44    | 148±39    | 12.7 |

SEM, standard error of means; DMI, dry matter intake; TMR, total mixed ration; SNF, solids-not-fat; d, day; SCC, somatic cell count.

Cows were fed corn silage TMR (periods 1 and 3) and Italian ryegrass silage TMR (period 2).
periods (Table 3). There were no differences in the plasma concentration of glucose, TG, T-CHO, and NEFA among the three experimental periods, while the BUN was higher (p<0.05) at period 2 (Table 4). The milk phytanic acid content was higher (p<0.05) at period 2 compared with that of periods 1 and 3 (Table 5). Also, the phytic acid content in feces was higher (p<0.05) in period 2 compared with that of periods 1 and 3. Phytol contents in feces had no differences among the three feeding periods (Table 5).

**DISCUSSION**

This experiment aimed to explore the conversion ratio of dietary phytol to milk phytanic acid in dairy cows fed TMR with different phytol contents. The absence of differences in DMI, milk yields, and milk composition among the periods indicates that the difference of silage source in TMR did not affect milk production performance due to the similar energy intake. The results are consistent with other reports [19,20].

The phytanic acid content in milk was higher for period 2. Schröder et al [4] reported that phytanic acid content in milk

### Table 3. Effects of feeding periods on fatty acid composition (% of total fatty acid) in milk of dairy cows

| Fatty acids | Period-1 | Period-2 | Period-3 | SEM |
|------------|----------|----------|----------|-----|
| C8         | 0.69±0.09 | 0.64±0.06 | 0.89±0.12 | 0.079 |
| C10        | 3.06±0.13 | 3.05±0.12 | 3.36±0.15 | 0.093 |
| C12        | 4.60±0.13 | 4.63±0.16 | 4.91±0.15 | 0.073 |
| C14        | 13.8±0.23 | 13.5±0.25 | 13.9±0.23 | 0.162 |
| C14:1      | 2.77±0.64 | 2.9±0.11  | 2.87±0.09 | 0.064 |
| C15        | 1.27±0.05 | 1.32±0.05 | 1.29±0.05 | 0.029 |
| C16        | 34.7±0.73 | 34.0±0.70 | 34.2±0.72 | 0.411 |
| C16:1      | 2.32±0.11 | 2.36±0.07 | 2.23±0.08 | 0.057 |
| C17        | 0.52±0.02 | 0.53±0.02 | 0.50±0.02 | 0.016 |
| C18        | 5.89±0.84 | 4.61±0.97 | 4.18±0.90 | 0.764 |
| trans-11 C18:1 | 3.42±0.21 | 6.10±0.07 | 5.72±0.20 | 1.315 |
| cis-9 C18:1 | 24.4±0.72 | 24.5±0.67 | 23.8±0.63 | 0.31 |
| cis-9, 12 C18:2 | 0.94±0.03 | 0.92±0.03 | 0.96±0.03 | 0.023 |
| cis-9, 12, 15 C18:3 | 0.59±0.03 | 0.67±0.03 | 0.67±0.04 | 0.035 |

SEM, standard error of means.

1) Cows were fed corn silage total mixed ration (periods 1 and 3) and Italian ryegrass silage total mixed ration (period 2).

### Table 4. Effects of feeding periods on plasma metabolite concentrations in dairy cows

| Item             | Period-1 | Period-2 | Period-3 | SEM |
|------------------|----------|----------|----------|-----|
| Glucose (mmol/L) | 3.98±0.07| 3.74±0.06| 3.99±0.07| 0.063 |
| TG (μmol/L)      | 63.9±3.08| 73.8±4.45| 70.8±5.45| 4.14 |
| T-CHO (μmol/L)   | 5.73±0.28| 5.81±0.28| 5.41±0.31| 0.257 |
| NEFA (μEq/L)     | 110.1±5.95| 100.3±4.71| 112.5±4.92| 5.1 |
| BUN (mmol/L)     | 1.96±0.10<sup>b</sup> | 2.72±0.10<sup>a</sup> | 1.98±0.08<sup>a</sup> | 0.109 |

SEM, standard error of means; TG, triglyceride; T-GHO, total cholesterol; NEFA, non-esterified fatty acids; BUN, blood urea nitrogen.

1) Cows were fed corn silage TMR (periods 1 and 3) and Italian ryegrass silage TMR (period 2).

<sup>b</sup> Means with different letters significantly differ (p<0.05).

### Table 5. Effects of feeding periods on milk and feces phytanic acid in dairy cows

| Item                        | n  | Period-1 | Period-2 | Period-3 | SEM |
|-----------------------------|----|----------|----------|----------|-----|
| Phytanic acid in milk (mg/kg)| 17 | 9.30±0.38<sup>b</sup> | 13.9±0.84<sup>a</sup> | 8.80±0.38<sup>b</sup> | 0.378 |
| Phytanic acid secretion in milk (mg/d) | 17 | 269.8±28<sup>b</sup> | 415.6±34<sup>a</sup> | 247.5±30<sup>b</sup> | 14.04 |
| Phytanic acid in faeces (mg/kg DM) | 4  | 1.15±0.04<sup>a</sup> | 1.65±0.04<sup>a</sup> | 1.17±0.03<sup>a</sup> | 0.022 |
| Phytol in faeces (g/kg DM) | 4  | 0.515±0.04 | 0.455±0.02 | 0.492±0.01 | 0.035 |

SEM, standard error of means; DM, dry matter; d, day.

1) Cows were fed corn silage total mixed ration (periods 1 and 3) and Italian ryegrass silage total mixed ration (period 2).

<sup>a</sup> Means with different letters significantly differ (p<0.05).
was between 0.021 and 0.2 mg/g milk. However, the phytanic acid content in this experiment was lower (9.3, 13.9, and 8.8 mg/kg for periods 1, 2, and 3, respectively) than that of their report, presumably due to the low phytol content in the TMR used in this study. In our experiment, silage and hay (Oats hay and alfalfa hay) accounted for 50% of TMR, while Schröder et al [5] used diets containing 86% of silage and hay. In addition, the feeding conditions and diets of cows were also important effective factors for milk quality [21]. The phytol intakes from TMR were calculated to be 9.5, 15.5, and 10.4 g/d for periods 1, 2, and 3 respectively. The phytol intake during period 2 was higher (p<0.05) compared with other periods. Also, the total phytanic acid secretion into the milk was calculated to be 0.27, 0.42, and 0.25 g/d for periods 1, 2, and 3 respectively. Based on the calculation, the conversion ratio of dietary phytol to milk phytanic acid was estimated to be only 2.6%. The phytanic acid content in feces was 1.2, 1.6, and 1.2 mg/kg for periods 1, 2, and 3, respectively. These results indicate that not all the phytanic acid produced in the rumen could be utilized by cows, and part of it is excreted into the feces. A slightly higher phytanic acid content in the feces was observed for period 2. This higher excretion was also affected by phytol intake. Because the total digestible nutrients content of TMR diets was about 70% for the three periods, DM digestibility of the TMR can be assumed to be 70%. Based on this assumption, fecal excretion of phytanic acid was estimated to be very small, only 0.07% of phytol intake. Thus, most of the dietary phytol was not recovered as phytanic acid in milk nor feces. This low appearance of phytanic acid was presumably owing to the low phytanic acid production in the rumen. In a previous study, the phytanic acid conversion ratio of IR silage with different phytol contents in the rumen was addressed; the conversion ratio of phytanic acid was only 15% to 36% and most phytol remained in the rumen [22], which was likely related to rumen microbial composition, diet composition, raising condition and feeding methods, etc. Previous studies demonstrated that dietary composition would affect the species and concentration of rumen microorganisms and consequently, affect phytanic acid production [23]. Therefore, under the condition of this study, most phytanic acid in the rumen might be utilized for purposes other than the milk component. Further studies on the factors affecting phytanic acid production in the rumen are necessary. In addition, phytol was found in feces, and there were no differences in the phytol content among the three periods. Using the above assumption, the fecal excretion of phytol was estimated to be 3.3, 2.8, and 2.9 g/d for periods 1, 2, and 3 respectively. This phytol excretion accounted for 35% (period-1), 19% (period-2), and 30% (period-3) of dietary phytol, respectively. Although the phytol intake of cows fed IR silage-based TMR was higher, the excreted ratio was lower for the cows fed IR. Compared with CS-based TMR, the apparent use of phytol in the total digestive tract seems to be higher for IR silage-based TMR. In this experiment, there were no differences in fatty acid profile in milk. The results are consistent with other reports [24]. Herbage is usually rich in C18:3 fatty acid [25] so that C18:3 in milk is one of the important fatty acid markers in some organic milk systems. The relationship between the C18:3 in milk fat and phytanic acid production was observed in some reports and showed that C18:3 was 3 times higher than phytanic acid [4]. However, no differences in C18:3 were found among the three feeding periods in this experiment. Although milk C18:3 could reflect the fatty acid composition in diets, it could not be regarded as a marker for phytanic acid content in milk.

The concentration of BUN in period 2 was higher than that in periods 1 and 3. The different BUN concentrations among the experimental periods might be due to a balance between hepatic production and output (urinary excretion and recycling) of urea-N [26]. BUN is affected by protein and energy consumed by animals and the breakdown of muscle protein [27]. In addition, it was reported that there was a positive correlation between BUN and ruminal ammonia [28,29]. Ruminal ammonia was utilized by rumen microorganisms [30]. Different components of diets or protein would affect ruminal ammonia content [31]. In this experiment, thus, the IR silage-based TMR would have a higher degradable N compared with the CS-based TMR.

In conclusion, the phytanic acid content in milk was higher for cows fed the IR silage-based TMR compared with the CS-based TMR. However, the conversion ratio of dietary phytol into milk phytanic acid was estimated to be only 2.6%. There were no differences in milk yield and milk composition contents between cows fed the IR silage-based TMR and CS-based TMR. Further studies are warranted on the factors affecting phytanic acid production in the rumen.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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REFERENCES

1. Szumacher-Strabel M, El-Sherbiny M, Cieslak A, Szczewochiak J, Winiarska H. Bioactive lipid components from
ruminant milk and meat: The new face of human health. In: Gupta VK, Tuohy M, editors. Biotechnology of bioactive compounds: sources and applications. Oxford, UK: John Wiley & Sons; 2015. pp. 599-629. https://doi.org/10.1002/9781118733103.ch25

2. Wanders RJA, Komen J, Ferdinandusse S. Phytanic acid metabolism in health and disease. Biochim Biophys Acta Mol Cell Biol Lipids 2011;1811:498-507. https://doi.org/10.1016/j.bbalip.2011.06.006

3. Roca-Saavedra P, Mario-Lorenzo P, Miranda JM, et al. Phytanic acid consumption and human health, risks, benefits and future trends: a review. Food Chem 2017;221:237-47. https://doi.org/10.1016/j.foodchem.2016.10.074

4. Schröder M, Farideh Y, Walter V. Investigating the day-to-day variations of potential marker fatty acids for organic milk in milk from conventionally and organically raised cows. Eur Food Res Technol 2011;232:167-74. https://doi.org/10.1007/s00217-010-1374-8

5. Schröder M, Nina LL, Ernest CT, Ensieh H, Farideh Y, Walter V. Phytanic acid concentrations and diastereomer ratios in milk fat during changes in the cow’s feed from concentrate to hay and back. Eur Food Res Technol 2012;234:955-62. https://doi.org/10.1007/s00217-012-1710-2

6. Halmemies-Beauchet-Filleau A, Kairenius P, Ahvenjärvi S, et al. Effect of forage conservation method on plasma lipids, mammary lipogenesis, and milk fatty acid composition in Italian ryegrass (Lolium multiflorum Lam.) silage. Anim Sci J 2020;91:e13309. https://doi.org/10.1111/asj.13309

7. Vanhatalo A, Kuoppala K, Toivonen V, Shingfield KJ. Effects of forage species and stage of maturity on bovine milk fatty acid composition. Eur J Lipid Sci Technol 2007;109:856-67. https://doi.org/10.1007/s10070-007-0023

8. Lv R, Elsabagh M, Obitsu T, Sugino T, Kurokawa Y, Kawamura K. Effects of nitrogen fertilizer and harvesting stage on photosynthetic pigments and phytol contents of Italian ryegrass silage. Anim Sci J 2017;88:1513-22. https://doi.org/10.1111/asj.12810

9. Lv R, Elsabagh M, Obitsu T, Sugino T, Kurokawa Y, Kawamura K. Effect of varying fermentation conditions with ensiling period and inoculum on photosynthetic pigments and phytol content in Italian ryegrass (Lolium multiflorum Lam.) silage. Anim Sci J 2020;91:e13309. https://doi.org/10.1111/asj.13309

10. Breese EL. Exploitation of genetic resource through breeding: Lolium species. In: McIver G, Bray RA, editors. Melbourne, Australia: Genetic Resources of Forage Plants CSIRO; 1983. pp. 275-88.

11. Shao T, Ohba N, Shimojo M, Masuda Y. Dynamics of early fermentation of Italian ryegrass (Lolium multiflorum Lam.) silage. Asian-Australas J Anim Sci 2002;15:1606-10. https://doi.org/10.5713/ajas.2002.1606

12. Chen L, Guo G, Yu C, Zhang J, Shimojo M, Shao M. The effects of replacement of whole-plant corn with oat and common vetch on the fermentation quality, chemical composition and aerobic stability of total mixed ration silage in Tibet. Anim Sci J 2015;86:69-76. https://doi.org/10.1111/asj.12245

13. AOAC. Official methods of analysis. 15th ed. Arlington, VA, USA: Association of Official Analytical Chemists; 1990. https://doi.org/10.1016/0165-9936(90)87098-7

14. Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. J Dairy Sci 1991;74:3583-97. https://doi.org/10.3168/jds.S0022-0302(91)78551-2

15. Sukhija PS, Painquist DL. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J Agric Food Chem 1988;36:1202-6. https://doi.org/10.1021/jf00084a019

16. Liljenberg C, Odham G. Gas chromatographic determination of phytol in plant material. Physiol Plant 1969;22:686-93. https://doi.org/10.1111/j.1399-3054.1969.tb07424.x

17. Takeda Y, Saito Y, Uchiyama M. Determination of phytochloride a, pyropheophorbide a and phytol. J Chromatogr A 1983;280:188-93. https://doi.org/10.1016/S0021-9967(00)91558-9

18. SAS Institute. SAS/STAT User’s Guide, Version 9.1 Edition. Cary, NC, USA: SAS Institute; 2004.

19. Rezaei J, Rouzbehyan Y, Zahedifar M, Fazaehli H. Effects of dietary substitution of maize silage by amaranth silage on feed intake, digestibility, microbial nitrogen, blood parameters, milk production and nitrogen retention in lactating Holstein cows. Anim Feed Sci Technol 2015;202:32-41. https://doi.org/10.1016/j.anifeedsci.2015.01.016

20. Paiva PG, Del Valle TA, Jesus EF et al. Effects of crude glycérin on milk composition, nutrient digestibility and ruminal fermentation of dairy cows fed corn silage-based diets. Anim Feed Sci Technol 2015;212:136-42. https://doi.org/10.1016/j.anifeedsci.2015.12.016

21. Blanch M, Carro MD, Ranilla MJ, Viso A, Vazquez-Anon A, Bach A. Influence of a mixture of cinnamaldehyde and garlic oil on rumen fermentation, feeding behavior and performance of lactating dairy cows. Anim Feed Sci Technol 2016;219:313-23. https://doi.org/10.1016/j.anifeedsci.2016.07.002

22. Lv R, Sato M, Elsabagh M, Obitsu T, Sugino T. Effect of fertilizer levels and harvesting stages of grass silage on ruminal phytanic acid production in vitro. The 10th Korea-Japan-China Joint Symposium on Rumen Metabolism and Physiology; 2015.

23. Belanche A, Fuente G, Pinloche E, Newbold CJ, Baleells J. Effect of diet and absence of protozoa on the rumen microbial community and on the representativeness of bacterial fractions used in the determination of microbial protein synthesis. J Anim Sci 2012;90:3924-36. https://doi.org/10.2527/jas.2011-
24. Razzaghi A, Valizadeh R, Naserian AA, Danesh Mesgaran M, Rashidi L. Effects of sucrose and sunflower oil addition to diet of Saanen dairy goats on performance and milk fatty acid profile. Livest Sci 2015;173:14-23. https://doi.org/10.1016/j.livsci.2014.12.002

25. Dierking RM, Kallenbach RL, Roberts CA. Fatty acid profiles of orchardgrass, tall fescue, perennial ryegrass, and alfalfa. Crop Sci 2010;50:391-402. https://doi.org/10.2135/cropsci2008.12.0741

26. Radostits OM, Gay CC, Blood DC, Hinchliffe KW. Veterinary medicine. A text book of the diseases of cattle, sheep, goats and horses. 10th ed. London, UK: Saunders Ltd; 2007.

27. Reist M, Erdin D, von Euw D, et al. Concentrate feeding strategy in lactating dairy cows: Metabolic and endocrine changes with emphasis on leptin. J Dairy Sci 2003;86:1690-706. https://doi.org/10.3168/jds.S0022-0302(03)73755-2

28. Huyen NT, Wanapat M, Navanukraw C. Effect of Mulberry leaf pellet (MUP) supplementation on rumen fermentation and nutrient digestibility in beef cattle fed on rice straw-based diets. Anim Feed Sci Technol 2012;175:8-15. https://doi.org/10.1016/j.anifeedsci.2012.03.020

29. West JW, Hill GM, Utley PR. Peanut skins as a feed ingredient for lactating dairy cows. J Dairy Sci 1993;76:590-9. https://doi.org/10.3168/jds.S0022-0302(93)77379-8

30. Cherdthong A, Wanapat M, Rakwongrit D, et al. Supplementation effect with slow-release urea in feed blocks for Thai beef cattle-nitrogen utilization, blood biochemistry and hematology. Trop Anim Health Prod 2014;46:293-8. https://doi.org/10.1007/s11250-013-0485-1

31. Ouellet DR, Chiquette J. Effect of dietary metabolizable protein level and live yeasts on ruminal fermentation and nitrogen utilization in lactating dairy cows on a high red clover silage diet. Anim Feed Sci Technol 2016;220:73-82. https://doi.org/10.1016/j.anifeedsci.2016.07.006