



# Preliminary study on the effect of season on urinary analytes in healthy Italian dairy cows

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## Abstract

**Background:** Season is known to affect serum analyte concentrations in dairy cows, and the same can be hypothesized for urinary analytes, but information in this regard is lacking.

**Objectives:** The aim of the study was to assess the effect of seasonality on urinary variables in healthy dairy cows.

**Methods:** Twelve Italian Holstein cows were randomly selected from a local herd, and urine specimens were collected once in each season of the year. For each cow, the lactation stage at each sampling time was also registered (lactation vs dry period), and physical examination and hematology and serum biochemistry measurements were used to assess health status. Three cows were voluntarily culled from the herd during the year; therefore, nine cows were included. Concentrations of urinary analytes recorded in each season for each cow were compared.

**Results:** Seasonality affected urinary protein-to-creatinine ratios ( $P = 0.012$ ) with lower median values in summer compared with spring ( $P = 0.020$ ) and autumn ( $P = 0.004$ ); differences were also found in urinary sodium-to-urinary creatinine ratio ( $P = 0.009$ ), with lower medians in summer compared with spring ( $P = 0.003$ ) and autumn ( $P = 0.020$ ). The direct consequences of higher summer temperatures and the acclimation strategies needed to adapt to this environment could explain the changes in urinary analytes that were recorded in the current study; in fact, decreased food and water consumption tend to increase urinary creatinine concentrations and decrease renal excretion of proteins and electrolytes.

**Conclusions:** The present results suggest that seasonality can affect urinary variables of healthy dairy cows.

## KEYWORDS

bovine, dairy cow, seasonality, urinalysis

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Over the last few years, routine biochemical profiles in dairy cattle have become useful diagnostic and prognostic tools for the assessment of productive and reproductive performances of livestock and herd animal welfare. Unlike blood analysis, urinalysis is a simple, safe, and noninvasive method to investigate health status; it creates no discomfort, poses no health-related risks, and has no direct side effects. Besides diagnosing specific kidney diseases or failures, the main indications for urinalysis in dairy cows are the detection and monitoring of energy balance and periparturient metabolic diseases, as highlighted by recent research.<sup>1,2</sup> Moreover, urine macro-mineral analysis is considered a very useful tool to gauge acid-base balances; urinalysis is more accurate than blood analysis in reflecting the attempts of the kidneys to stabilize the serum acid-base status, which is subject to strict homeostatic control.<sup>2</sup> Thus, urinalysis could be a useful tool to troubleshoot and monitor metabolic problems, but information on values in healthy cows and the influence of external factors is essential for the correct evaluation of results. In animals, a large number of factors, such as species, type or breed, sex, age, nutritional, and health status, as well as seasonal and physiologic variations, including pregnancy and lactation, are known to affect serum biochemistry and mineral levels. Regarding urinalysis, preanalytic and analytic factors have been reported to affect results for other species, especially dogs,<sup>3</sup> but similar studies on the bovine species have not thus far been published. Except for two recent studies focusing on urinalysis and urinary protein-to-creatinine ratios,<sup>4,5</sup> reference values for urinary variables in cows are outdated,<sup>6</sup> especially in view of the fast changes in dairy cattle genetics and productiveness, and irrespective of season, which is already known to affect blood analyte concentrations.<sup>7,8</sup> Seasonality has been proven to affect hematologic variables mainly because of the summer heat stressors and sweating.<sup>8,9</sup> Considering the central role of the kidney in maintaining homeostasis, an influence of seasonality on urinary variables in dairy cows can be hypothesized. Therefore, the present study aimed to investigate possible urinary variable changes in healthy Holstein cows from Northern Italy, according to season.

The study was conducted on a dairy farm located in Northern Italy. The herd consisted of approximately 100 Italian Holstein cows with an average milk production per lactation (305 days) of 7780 kg/cow (3.64% milk fat, 3.32% milk protein). The cows were housed in barns with sand-bedded free-stall pens and fed using a total mixed ratio (TMR) system. Twelve healthy cows were randomly selected from the herd and repeatedly sampled during the different seasons, as reported below. Health status was clinically assessed through veterinary examinations performed on the day of each sampling and confirmed later by hematology and serum biochemistry analyses, not included as results in this study. At each sampling time, cows were registered as belonging to lactation or dry period groups.

For all the cows, urine and blood specimens were collected as part of a routine health screening, specifically requested by the farmer. Therefore, formal approval from the Ethical Committee was not required (EC decision October 29, 2012, renewed with the protocol n° 02-2016).

Urine specimens were collected four times during a 1-year period: once in winter (February), spring (May), summer (July), and autumn (November), always during the morning. Locally registered mean monthly temperatures were 6°C in February, 19.6°C in May, 25.4°C in July, and 8.2°C in November. At each sampling time, 10 mL of midstream urine were collected from spontaneous micturition or by tickling the perineum to stimulate urination into appropriate sterile containers and promptly transferred under refrigeration for urinalysis to the Central Laboratory of the Veterinary Teaching Hospital of the University of Milan (Lodi, Italy) located 3 km from the herd. The samples were processed within 2 hours of collection. For processing, 5 mL of urine were transferred into conical tubes and subjected to a chemical-physical examination. After visual assessment of appearance and color, the pH was measured with a bench pH meter (pH 211 Microprocessor pH Meter, Hanna Instruments),<sup>10</sup> and tubes were centrifuged at 500g for 10 min. Urinary specific gravity (USG) was assessed on supernatant with a manual temperature-compensated refractometer (Model Refractometer 105, Sper Scientific), and three aliquots of supernatant (1.5 mL each) were frozen at -20°C until biochemical analysis (performed within 1 week).<sup>11</sup> The resulting pellet was resuspended in the remaining 500 µL of supernatant, and 50 µL of the obtained suspension were placed on a slide for microscopic examination of unstained sediment at low (100×) and high (400×) magnification. The presence of bacteria, crystals, casts, lipid droplets, and epithelial cells was recorded at low-power microscopic fields on a -/+ /++ scale and were always performed by the same operator. The presence of erythrocytes and leukocytes was quantified, calculating the average numbers of cells in 10 high-magnification fields. Specimens with at least one of the following abnormalities on microscopic evaluation, bacteria, crystals, casts, lipid droplets, epithelial cells, erythrocytes, or leukocytes, were registered as positive specimens.

Urine analyte concentrations were determined on an automated BT3500 clinical chemistry instrument (Biotecnica Instruments). Each method was subjected to daily quality control applications using two levels of control sera consisting of human serum with added bovine plasma supplied by the company FUTURLAB S.R.L. Urinary proteins (uP, mg/L) were measured using a colorimetric method based on pyrogallol red (Urine protein, Sentinel diagnostics). This method was subjected to quality control before the analysis, using two levels of urine control consisting of human salivary amylase, hCG (human Chorionic Gonadotropin) derived from human urine, and human and bovine serum albumin, supplied by the company FUTURLAB S.R.L. The concentration of urinary creatinine (uC) (mg/L, modified Jaffé method) was measured after the supernatant was diluted 1:100 with demineralized water, and the urinary protein-to-creatinine ratio (uP/uC) was subsequently calculated. Urinary sodium (uNa, mmol/L) and potassium (uK, mmol/L) concentrations were determined using an indirect ion-selective electrode method. Urinary calcium (uCa) (mmol/L, colorimetric o-cresolphthalein complexone method), and magnesium (uMg) (mmol/L, enzymatic kinetic method based on the formation of phosphogluconic acid and NADPH) were determined in urine supernatants using reagents supplied by the same company of

the control sera. For uNa, uK, uCa, and uMg, the ratios with uC (uNa/uC, uK/uC, uCa/uC, and uMg/uC) were calculated after the conversion of uC to mmol/L.

Statistical analysis was performed with the Analyze-it software (Analyze-it software, Leeds, UK). Differences for each analyte were analyzed according to season by a nonparametric ANOVA test for independent data (Kruskal-Wallis test) followed by a Bonferroni test to evaluate paired differences. The significance level was set at  $P < 0.05$ .

From the initial group of 12 cows, nine cows were included in the study, as three cows were voluntarily culled by the farmer for economic reasons during the year. Regarding the chemical-physical examination, all specimens showed a straw yellow-to-gold color on visual inspection, except for one specimen that appeared pinkish due to blood contamination. The appearance was often clear or almost clear, although some specimens showed increased turbidity. USG values ranged between 1.013 and 1.043, with no differences among seasons ( $P = 0.290$ ). No seasonal differences were detected for pH values ( $P = 0.120$ ) (min-max values: 7.8–8.6). Leukocytes (present in eight of 36 specimens) ranged from a minimum of 0/high power field (hpf) to a maximum of 20/hpf. Chemical-physical and microscopic features of urine specimens in the four seasons are summarized in Table 1. Results about the median values of urinary analytes according to the different seasons are reported in Figures 1 and 2. Differences were found for uP/uC ( $P = 0.012$ ), with lower median values in summer compared with spring ( $P = 0.020$ ) and autumn ( $P = 0.004$ ) (Figure 1), and for uNa/uC ( $P = 0.009$ ) with lower medians in summer compared with spring ( $P = 0.003$ ) and autumn ( $P = 0.020$ ) (Figure 2).

Previous studies demonstrated that seasonality could induce variations in the hematologic and metabolic profiles of dairy cows.<sup>8,9</sup> Our hypothesis was that seasonality also could have an influence on urine composition; albeit with the limitation posed by the small sample size, the results of the present study support this hypothesis, as significant changes in some urinary variables were detected among the seasons.

The spot specimen urine method might introduce bias in evaluating urinary excretion because diurnal and day-to-day variations in urine volume can affect the concentrations of many variables. Nevertheless, Chizzotti et al<sup>12</sup> stated that the spot sampling

technique could be used to estimate the daily urinary excretion of substances in Holstein cattle under practical conditions, where total urine collection is unfeasible. Since daily creatinine production and consequent excretion are proportional to body mass, measuring body weight together with uC should accurately estimate the average urinary output of nutrients.<sup>13</sup> However, as the proportion of tissues varies in growing animals, there may be variations in the daily creatinine excretion expressed as a function of muscle mass variation and practically reflected by body weight measurements; in fact, the rate of creatinine excretion per unit of body weight varies considerably among cows,<sup>13</sup> causing individual cow urine output to differ substantially from the estimated output. As creatinine is excreted in urine at a constant rate, uC can be used to standardize urine analyte concentrations for fluctuations due to the hydration level.<sup>14</sup> Therefore, we decided to estimate electrolyte outputs only through the normalization to uC.

Summer differed from the other seasons, as characterized by lower uNa/uC and uP/uC ratios compared with spring and autumn. Sweating is a major thermoregulatory mechanism to dissipate excess body heat when lactating cows are exposed to a hot environment. Thus, with high temperatures, cattle present an energy expense for thermoregulation and a parallel reduction of food intake. This acclimation strategy can explain the changes in urinary analytes recorded in our study. Looking at the present data, uC and USG tended to be higher in summer compared with all other seasons, but not significantly. The increasing tendency of uC and USG levels in summer is probably attributable to the changes in urine volume, as reported by Lee et al.<sup>15</sup> These variations in urine volume may be augmented in the summer season, due to sweating and decreased feed and water uptake; consequently, water reabsorption from the kidney increases, resulting in a higher uC concentration. Nevertheless, the uC differences found among the different seasons were not statistically significant, likely due to the low number of observations, masking a possible significant difference despite an evident trend in median values. Therefore, the lower uNa/uC and uP/uC ratios detected in summer are likely attributable to different degrees of renal sodium and protein excretion. To date, specific reference intervals for the uP/uC in the bovine have only been reported in two recent studies.<sup>4,5</sup> The uP/

TABLE 1 Results from the chemical-physical and microscopic examination of urine specimens for each season

	Winter	Spring	Summer	Autumn
pH (min-max)	8.1–8.4	8.1–8.4	7.8–8.4	8.1–8.6
USG (min-max)	1.024–1.028	1.013–1.035	1.020–1.043	1.020–1.042
Erythrocytes	0/9 <sup>a</sup>	0/9 <sup>a</sup>	0/9 <sup>a</sup>	1/9 <sup>a</sup>
Leukocytes	3/9 <sup>a</sup>	1/9 <sup>a</sup>	3/9 <sup>a</sup>	1/9 <sup>a</sup>
Epithelial cells	2/9 <sup>a</sup>	1/9 <sup>a</sup>	0/9 <sup>a</sup>	2/9 <sup>a</sup>
Crystals (struvite)	1/9 <sup>a</sup>	2/9 <sup>a</sup>	1/9 <sup>a</sup>	1/9 <sup>a</sup>
Bacteria (cocci)	2/9 <sup>a</sup>	1/9 <sup>a</sup>	1/9 <sup>a</sup>	0/9 <sup>a</sup>

<sup>a</sup>Positive specimens/total specimens.

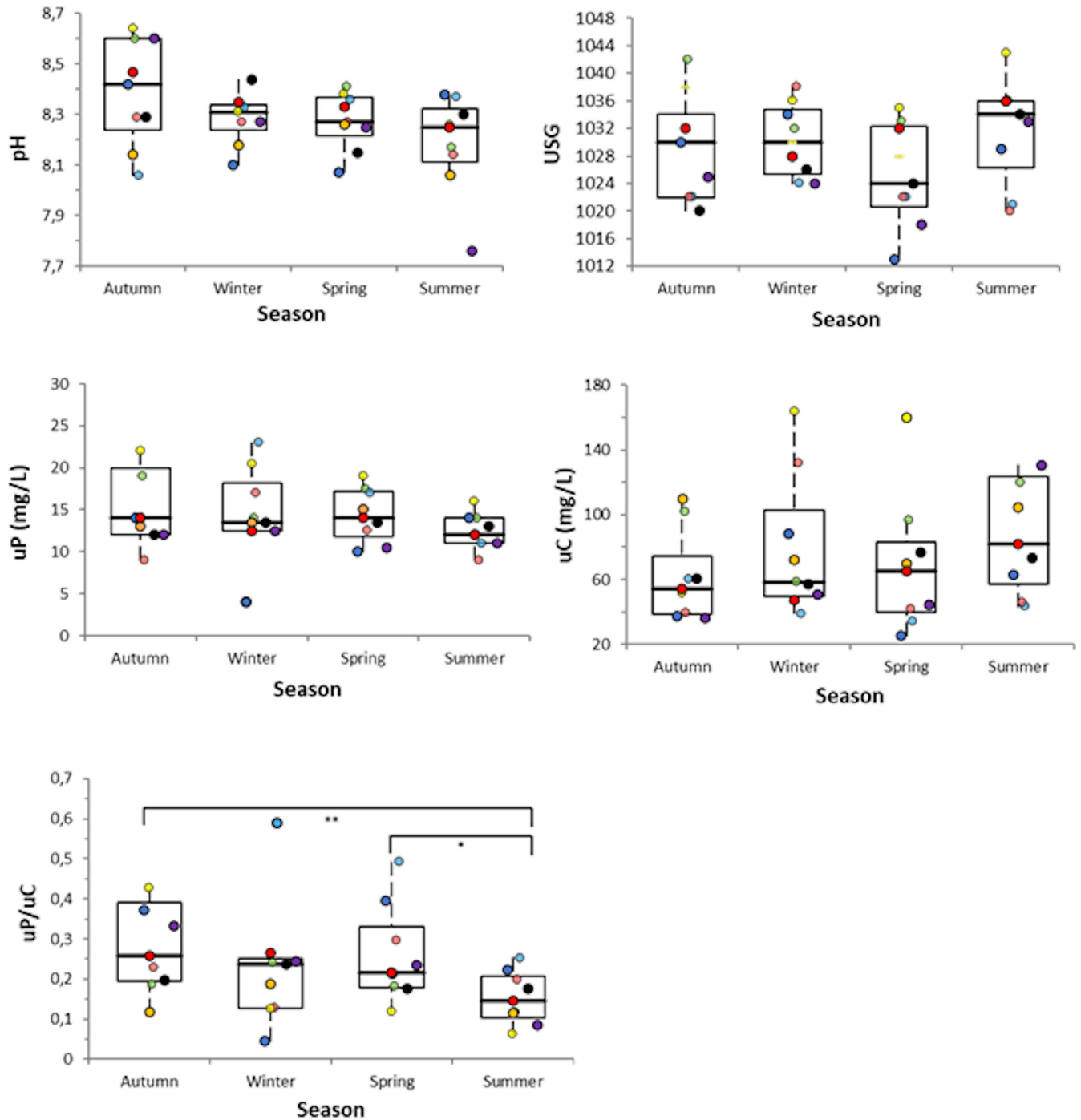


FIGURE 1 Changes in urinary pH, specific gravity, protein and creatinine concentrations, and urinary protein to creatinine ratios in dairy cows among the different seasons. The boxes indicate the I-III interquartile range (IQR), the horizontal line the median value, and the whiskers extend to further observation within quartile I minus  $1.5 \times$  IQR or to further observation within quartile III plus  $1.5 \times$  IQR; + indicate the near outliers (ie, values higher than the quartile III plus  $1.5 \times$  IQR); each color denotes a single cow; \*, \*\*: indicate differences among seasons ( $P < 0.05$ ,  $P < 0.01$ , respectively)

uC values recorded in the present research are consistent with the study by Herman et al<sup>4</sup>; these authors also found that proteinuria in cows was not associated with other factors, such as breed or diet, so the effect of changes in food composition on the UPC ratio over time should be excluded. Katsoulou et al<sup>5</sup> reported that a uP/uC ratio of less than 0.19 is associated with an absence

of renal damage, whereas higher values raise suspicion for renal disease. However, the same author stated that, although the 0.19 uP/uC ratio can represent an optimal cut-off point to differentiate animals with and without renal lesions, it cannot be used as an indicator due to the low sensitivity and specificity. In the present study, median uP/uC ratios were above this cut-off (0.22 in spring;

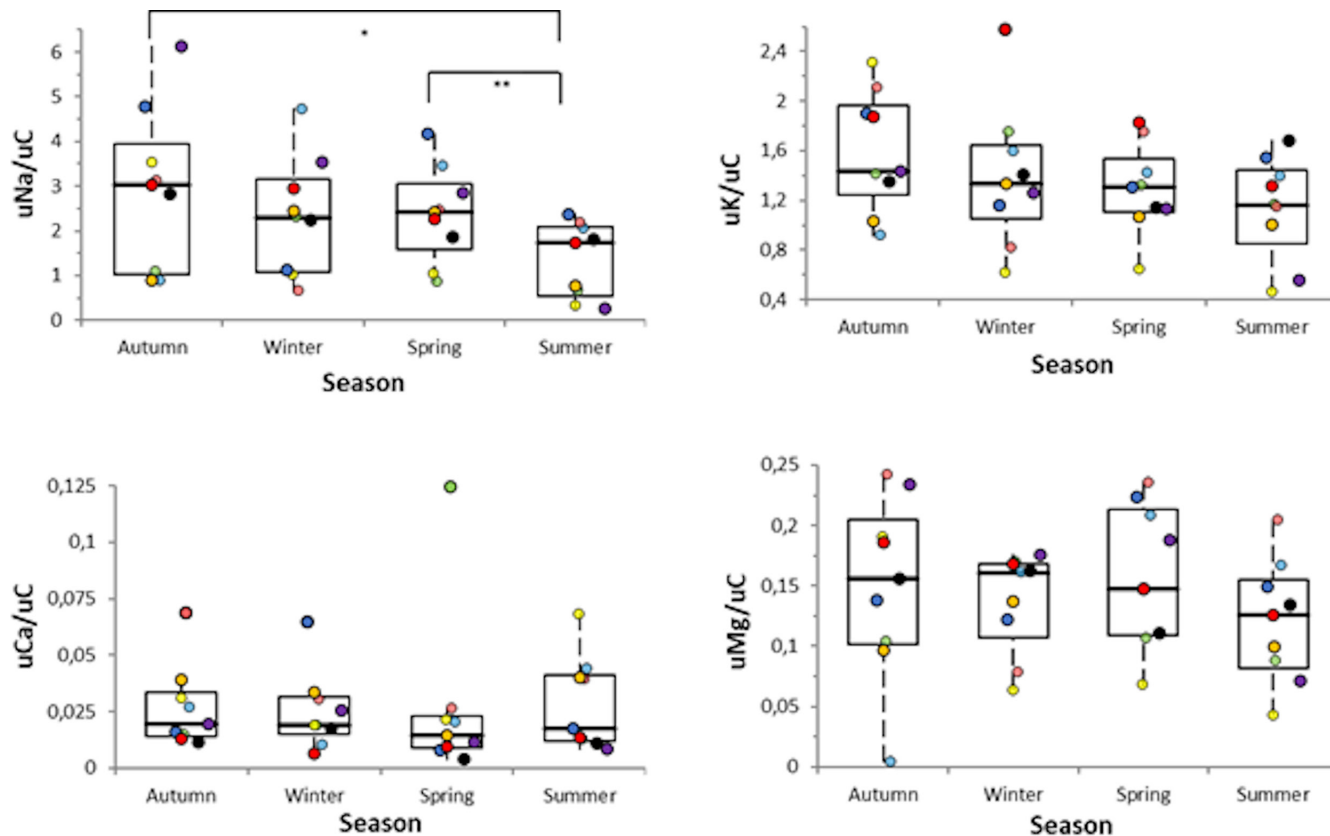


FIGURE 2 Changes in urinary Na/C, K/C, Ca/C, and Mg/C ratios in dairy cows among the different seasons. The boxes indicate the I-III interquartile range (IQR), the horizontal line the median value, and the whiskers extend to further observation within quartile I minus  $1.5 \times$  IQR or to further observation within quartile III plus  $1.5 \times$  IQR; + indicate the near outliers (ie, values higher than the quartile III plus  $1.5 \times$  IQR); each color denotes a single cow; \*, \*\*: indicate differences among seasons ( $P < 0.05$ ,  $P < 0.01$ , respectively)

0.26 in autumn; 0.24 in winter) except for in the summer period (median uP/uC = 0.15), thus confirming the influence of seasonality on this variable.

In the current study, the stage of lactation for the nine cows was also registered at each sampling time; most of the specimens were taken from lactating cows, while seven of the total 36 specimens were taken from dry cows, and only one of these was performed in summer. When exploring variations of urinary variables in lactating dairy cows, the influence of the production stage should also be considered, as the stage of lactation is already known to influence serum analyte concentrations in cows.<sup>7,8,16</sup> The limited number of cows enrolled in the present study did not allow us to clarify this aspect, which thus requires further investigations.

The mean urinary pH was always  $>8$ , in agreement with those reported for healthy cows.<sup>14</sup> According to Roche et al,<sup>17</sup> the urine pH of dairy cows is generally between 8.0 and 8.5 throughout the year but can be lowered by a very low dietary cation-anion difference. Seasonal changes in TMR compositions could have also occurred in the present study, but urinary pH remained stable throughout the seasons and always fell in the physiologic range for cattle.<sup>4,5,17</sup>

The finding of struvite crystals and epithelial cells in the urinary sediment of healthy cows, which were observed in five of 36 total

specimens, has already been reported.<sup>5</sup> Regarding leukocytes, in view of the absence of clinical signs attributable to urinary infection and of the urine collection method used (spontaneous micturition), the presence of few leukocytes in urinary sediments was considered as a contamination from the reproductive or gastrointestinal tract (half of the urinary sediments with leukocytes were taken from postpartum cows) with no pathologic significance.

A limitation of this study is that, for ethical reasons, sampling could not be repeated over time within each single session. Theoretically, this lack of information does not allow one to exclude that the changes observed among the different seasons were actually due to inherent intra-seasonal variability on the urine analytes. However, this latter hypothesis seems to be unlikely since the primary changes were recorded in summer when environmental conditions (temperature, humidity) could explain the observed differences compared with the other seasons. The uniformity of the cows enrolled was optimal to rule out any possible confounding effects of herd, food composition, and climatic variation, but it can also represent a limitation. Moreover, cow-to-cow variation of the creatinine coefficients could be reduced by increasing the number of cows enrolled.<sup>15</sup>

In conclusion, urinalysis is a useful and noninvasive examination that is rarely used in bovine medicine, which is partly because many practitioners consider it a strenuous procedure, but mainly because

there is a lack of reference data for this species. To our knowledge, this is the first study showing changes in urinary analytes of dairy cows according to seasonality. Our preliminary results support the development of future studies designed to confirm the urinary reference intervals specific for each season and lactation stage in dairy cows.

#### ACKNOWLEDGEMENTS

The article is funded by Università degli Studi di Milano. [Correction added on 11 May 2023, after first online publication: CRUI-CARE funding statement has been added.]

#### DISCLOSURE

The authors have indicated that they have no affiliations or financial involvement with any organization or entity with a financial interest in, or in financial competition with, the subject matter or materials discussed in this article.

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**How to cite this article:** Probo M, Giordano A, Rocca V, Moretti P, Paltrinieri S. Preliminary study on the effect of season on urinary analytes in healthy Italian dairy cows. *Vet Clin Pathol*. 2022;51:408–413. doi: [10.1111/vcp.13115](https://doi.org/10.1111/vcp.13115)