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RESEARCH ARTICLE

# Preliminary Study on Urine Chemistry and Protein Profile in Cows and Heifers

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**ABSTRACT**

Urinalysis offers important clinical information regarding not only the kidney function but also the general health status of an organism. The aims of this research were to obtain preliminary data on urine chemistry and electrophoretic protein profile from cows and heifers, to compare electrophoretic profiles of not pregnant with pregnant animals and to evaluate their changes as the pregnancy progresses. Eight heifers and six cows were included in the study and 97 urine samples were collected. Complete urinalysis was performed and urinary proteins were separated by electrophoresis. Considering the pregnancy as a source of variability, significant differences were reported between pregnant and not pregnant heifers for the urine specific gravity (P=0.005), urine total proteins (P=0.009) and urine total proteins to urine creatinine ratio (UPC) (P=0.008). The majority of urine samples analysed in this study showed common protein bands. A mean of protein bands of 17±3 was detected in heifers, while a mean of 13±3 protein bands was recorded in cows. The putative proteins were uromodulin, transferrin, albumin, heavy and light chains of immunoglobulins. The comparison between pregnant and not pregnant animals showed qualitative differences, with the absence of three bands in not pregnant cows including the putative alpha-fetoprotein. In conclusion, urinalysis is an economical and a non-invasive diagnostic protocol, which should be routinely used for the clinical evaluation of large animals. The data reported in the present study could be considered suggestive of healthy animals and they confirmed those previously reported in the literature.



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## INTRODUCTION

By the evaluation of color, transparency, chemical composition and urinary sediment, urinalysis offers important clinical information regarding not only the kidney function but also the general health status or physiological condition (e.g. pregnancy) of an organism (Rawat *et al.*, 2016; Piech and Wycislo, 2019). Of the myriad of the urinary molecules, proteins are of great interest due to their role in pathophysiological processes. The importance of the study of the urinary proteome was recently highlighted in farm and companion animals (Miller *et al.*, 2014; Ferlizza *et al.*, 2015; Isani *et al.*, 2018).

In particular, urine reference values of cattle, as well as the composition of the bovine urinary proteome have been previously reported (Pyo *et al.*, 2003; Bathla *et al.*, 2015; Mokbul *et al.*, 2016; Rawat *et al.*, 2016; Herman *et al.*, 2019). These studies aimed at the discovery of biomarkers, which could help the farmer in the early pregnancy diagnosis as well as in the detection of the parturition's onset. An early pregnancy diagnosis is essential for the reduction of the interval between the gestation and the reintroduction of cows in the breeding program (Mokbul *et al.*, 2016). Moreover, the determination of the onset of parturition is important to give proper care of calves, avoiding complications during the parturition (Wawrzykowski *et al.*, 2018). Consequently, many studies have been focused on pregnancy, given its key role in the dairy industry, while few researches projects have been performed to identify the physiological urinary proteins of cattle. Indeed, the urinary proteome of farm and companion animals was studied to a lesser extent than the human proteome (Adachi *et al.*, 2006; Candiano *et al.*, 2010; Brandt *et al.*, 2014; Almeida *et al.*, 2015; Ferlizza *et al.*, 2015; Rawat *et al.*, 2016; Isani *et al.*, 2018). Different protocols have been proposed for the study

of the urinary proteome, including one and two-dimensional gel electrophoresis followed by the identification of the most important proteins by mass spectrometry. Considering the paucity in the literature of studies on cow urine characterization, the aims of this research were to obtain preliminary data on urine chemistry and electrophoretic protein profile from cows and heifers, to compare electrophoretic profiles of not pregnant with pregnant animals and to evaluate their changes as the pregnancy progresses.

## MATERIALS AND METHODS

**Study subjects and sample collection:** Ninety-seven urine samples were obtained from eight heifers (62 samples) and six cows (35 samples) hosted at the farm of the Department of Veterinary Medical Sciences (University of Bologna) (Table 1). The barn was equipped with automated cooling systems and made up of a resting area with berth-barns with straw, arranged in double row system tail to tail. The floor was made up of striped concrete and was cleaned with automatic scraper systems. The heifers and the cows during the dry period were allocated in the part of the barn with a permanent sloping bedding. The stable was provided with an advanced system for the electronic control of the herd and a new generation milking room equipped with on-line analyser of milk quality (indexes analysed: fat, protein, lactose, urea, somatic cells and electrical conductivity). Moreover, an automatic feeding system, an electronic scale to automatically weighed animals at each milking, and a modern horizontal chopper-mixer wagon were also present. The average production of animals per lactation was 10,500 kg of milk with 3.8% fat and 3.4% protein.

Urine sampling was performed every month, at 8 am, from December 2011 to November 2012. Twenty ml of mid-stream urine were collected by manual stimulation of the vulva in urine cups (Goff *et al.*, 2014). Animals were in a good health status based on periodic routine physical examination and blood analyses and were not affected by diseases during this study.

**Urinalysis, urine chemistry and urine protein to creatinine ratio calculation:** The samples were subjected to routine urinalysis, including urine specific gravity (USG), semi-quantitative dipstick test (Dirui® H10 Urine Analysis Strips; Dirui analyser H-500), centrifugation at 1,500 *g* for 10 minutes and microscopic sediment analysis (Ferlizza *et al.*, 2015; 2017). The supernatants were stored at -80°C for subsequent analyses. The microscopic sediment analysis was performed within 5 hours from collection. Two drops (30 µL each) of sediment (uncoloured and coloured with fuchsine) were examined under microscopic low power field (100X) and high-power field (400X) searching for the presence of casts, crystals, urinary tract cells, red blood cells and leucocytes.

On urine supernatants, urine total proteins (uTP) and urine creatinine (uCrea) were determined using commercial kits (Urinary/CSF Protein, OSR6170, and Creatinine OSR6178, Olympus/Beckman Coulter) on an automated chemistry analyser (AU 400, Olympus/Beckman Coulter). The uTP to uCrea ratio (UPC) was calculated using the following formula: UPC = uTP (mg/dL)/uCrea (mg/dL).

**SDS-PAGE:** Eighty-four samples were subjected to sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as previously reported (Ferlizza *et al.*, 2015; Isani *et al.*, 2016). Two micrograms of urine proteins were separated on precast gels (10%, 12%, 4-12%; NuPAGE ®, Thermo Fisher Scientific, Waltham, Massachusetts, USA) in buffers containing SDS (MES; MOPS; Thermo Fisher Scientific) and reducing conditions. The gels were stained with silver staining (SilverQuest™ Staining Kit Invitrogen), then digitalised and the pherograms were obtained using GelAnalyzer 2010 software.

**Statistical analysis:** Statistical analysis was performed using the software R® (Rx64 3.4.3) and RStudio. The normal distribution of the data was tested by the Shapiro-Wilk normality test. A $P>0.05$ was considered indicative of a normal distribution. Data are reported as mean±standard deviation or median and range (minimum-maximum value) depending on normal or not normal distribution, respectively. Mean values of repeated measures from the same animal were calculated (Petrie and Watson, 2013) in each group (cows vs heifers) and/or condition (pregnant vs not pregnant).

Normally distributed variables were analysed by t-test to determine the differences between heifers and cows and, within each group, the differences between the pregnant and not pregnant animals. The variables without normal distribution were tested by Wilcoxon rank sum test. The significance level was set at $P<0.05$.

## RESULTS

**Urinalysis:** *Heifers* - Heifers were 1-2 years old and, out of eight individuals, seven were pregnant. Three specimens (ID: 266, 283 and 290) were followed throughout the pregnancy and one of them (ID: 290) was sampled also at the time of oestrus. The other three heifers (ID: 236, 247 and 250) were followed in the second and third trimester of pregnancy as well as in the post-partum period. One heifer (ID: 266) was followed starting from before the pregnancy up to the 232nd day. One heifer (ID: 296) was never pregnant and another one (ID: 283) had a miscarriage in the sixth month of the first pregnancy and, consequently, the next gestation was monitored.

All urine samples were clear and pale yellow colour. Urine pH ranged from 8.5 to 9, while the USG had a mean value of 1.032±0.002. Complete data are reported in Table 2.

The semi-quantitative dipstick test resulted positive for proteins (30-500 mg/dL) in 59 samples, for erythrocytes (50 Ery/µL) in one sample, for ketones (150 mg/dL) in one sample collected 24 days after the parturition, and negative in all samples for leucocytes, glucose and bilirubin. The microscopic evaluation of urine sediments revealed epithelial cells in all the samples analysed, while bacteria and casts (hyaline, granular and fatty) were present in three urine samples. Crystals were found in 15 urine samples, in particular, eight samples showed triple phosphate crystals, six samples calcium phosphate crystals and two urines calcium carbonate crystals (Fig. 1).

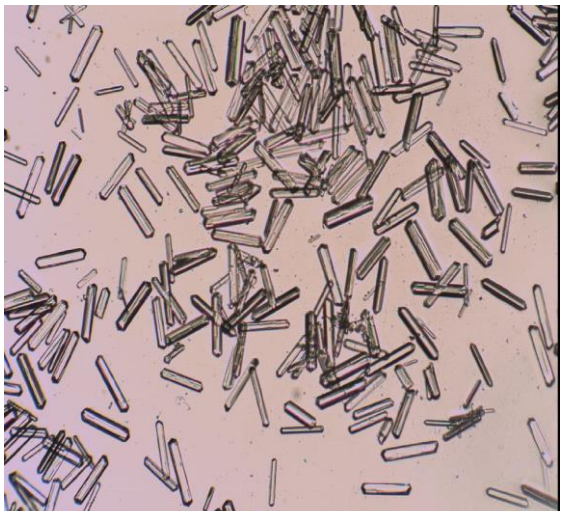

**Fig. 1.** Phosphate crystals in the microscopic urine sediment of a heifer (100x).

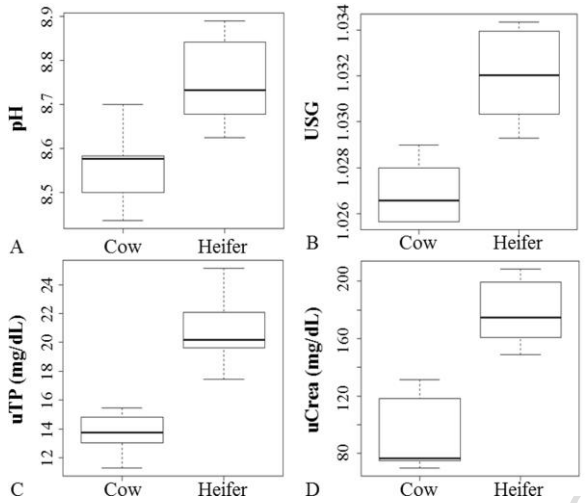


**Fig. 2:** Comparison between cows and heifers for pH (A), urine specific gravity (USG) (B), urine total proteins (uTP mg/dL) (C) and urine creatinine (uCrea mg/dL) (D).

*Cows* - Cows were 2-5 years old and, out of the six animals, five were pregnant. Three specimens (ID: 61, 62 and 185) were followed through the pregnancy up to the post-partum period and two cows (ID: 191 and 193) from the second and third month of pregnancy up to the seventh and eighth month, respectively. Two animals were sampled also at oestrus (ID: 61 and 242), one of them (242) was never pregnant.

Cow urines appeared light to medium yellow and clear, with a urine pH ranging from 8.5 to 9. The USG had a mean value of 1.027±0.001. Complete data are reported in Table 2. Dipstick test results were negative for erythrocytes, leukocytes, glucose and bilirubin in all samples, while positivity to proteins was recorded in 30 samples analysed (30-100 mg/dL). Two samples presented positivity to ketones (15 mg/dL) in urines collected 13 days and 33 days after the parturition. The microscopic examination of urine sediment revealed rare epithelial cells.

Considering the comparison between cows and heifers, significant differences were found for pH (P=0.003), USG (P=0.0001) (Fig. 2) and proteins mg/dL (P=0.003). USG was significantly different also between pregnant and not pregnant heifers (P=0.005) (Fig. 3).

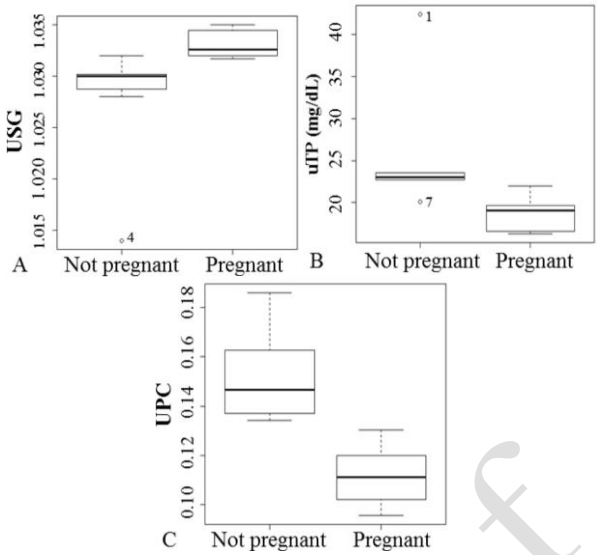


**Fig. 3:** Comparison between pregnant and not pregnant heifers for the urine specific gravity (USG) (A), urine total proteins (uTP mg/dL) (B) and urine protein to urine creatinine ratio (UPC) (C). Circles and numbers in the box-plots (A and B panels) indicate the outlier specimens.

**Table 1:** Animals' breed, group, age and lactation number

| ID | Bovine breed | Group | Age | Lactation* |
|---|---|---|---|---|
| 61 | Holstein-Friesian | Cow | 5-y and 2-mo | 3rd |
| 62 | Holstein-Friesian | Cow | 5-y and 2-mo | 3rd |
| 185 | Holstein-Friesian | Cow | 3-y and 11-mo | 1st |
| 191 | Holstein-Friesian | Cow | 3-y and 7-mo | 2nd |
| 193 | Holstein-Friesian | Cow | 3-y and 5-mo | 2nd |
| 242 | Holstein-Friesian | Cow | 2-y and 3-mo | 1st |
| 236 | Holstein-Friesian | Heifer | 2-y and 4-mo | 0 |
| 247 | Holstein-Friesian | Heifer | 2-y | 0 |
| 250 | Holstein-Friesian | Heifer | 1-y and 10-mo | 0 |
| 251 | Holstein-Friesian | Heifer | 1-y and 10-mo | 0 |
| 266 | Holstein-Friesian | Heifer | 1-y and 7-mo | 0 |
| 283 | Holstein-Friesian | Heifer | 1-y and 5-mo | 0 |
| 290 | Holstein-Friesian | Heifer | 1-y and 2-mo | 0 |
| 296 | Holstein-Friesian | Heifer | 1-y and 2-mo | 0 |

*At the time of the start of the study.

**Table 2:** Urinalysis (USG, pH and dipstick), urine total proteins (uTP), urine creatinine (uCrea) and UPC of cows and heifers. Data are reported as mean±SD or median and range (min - maximum values) depending on normal or not normal distribution, respectively

| Variable | Cows (N=6; n=35) | Heifers (N=8; n=62) | *P** |
|---|---|---|---|
| USG | 1.027±0.001 | 1.032±0.002 | 0.0001 |
| pH | 8.56±0.08 | 8.75±0.09 | 0.003 |
| Leu (cell/µL) | 0 | 0 | NA |
| Pro (mg/dL) | 62.1±20.5 | 120.6±37.0 | 0.003 |
| Glu (mg/dL) | 0 | 0 | NA |
| Ket (mg/dL) | 0 (0-5) | 0 (0-21.4) | 0.5 |
| Bil (mg/dL) | 0 | 0 | NA |
| Ery (cell/µL) | 0 | 0.9±2.5 | 0.5 |
| uTP (mg/dL) | 13.7±1.6 | 20.8±2.6 | 0.0005 |
| uCrea (mg/dL) | 94.3±28.4 | 177.7±23.4 | 0.0008 |
| UPC | 0.16±0.04 | 0.12±0.01 | 0.09 |

N = number of specimens; n = number of urine samples; USG = urine specific gravity; Leu= Dipstick Leukocytes; Pro = Dipstick Proteins; Glu = Dipstick Glucose; Ket = Dipstick Ketones; Bil = Dipstick Bilirubin; Ery = Dipstick Erythrocytes; uTP = urine total proteins; uCrea = urine creatinine; UPC = urine protein to urine creatinine ratio; NA = not applicable. * *P* refers to the comparison between cows and heifers.

**Urine total proteins, urine creatinine and UPC:** Complete data are reported in Table 2. Mean values for uTP, uCrea and UPC in heifer urine were 20.8±2.6 mg/dL, 177.7±23.4 mg/dL and 0.12±0.01, respectively. Mean values for uTP, uCrea and UPC in cow urine were 13.7±1.6 mg/dL, 94.3±28.4 mg/dL and 0.16±0.04, respectively.

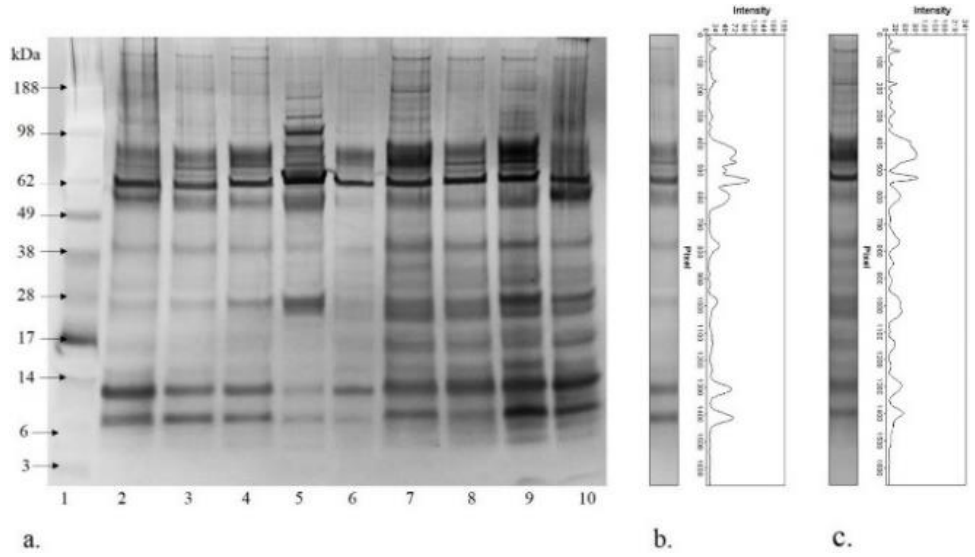


**Fig. 4a:** Representative gel of urine samples from heifers and cows. Lane 1: molecular weight marker. Lane 2: urine samples from cows in post-partum period. Lane 3, 4: urine samples from pregnant cows. Lane 5 and 10: urine samples of not pregnant heifers. Lane 6-9: urine samples from pregnant heifers. **b.** Representative pherogram of a urine sample of cow. **c.** Representative pherogram of a urine sample of heifer.

**Table 3:** USG, pH, urine total protein (uTP), urine creatinine (uCrea) and UPC of pregnant and not pregnant animals. Data are reported as mean±SD or median and range (min - max values) depending on normal or not normal distribution, respectively

| Variable | Heifers (N=8; n=62) | | Cows (N=6; n=35) | |
|---|---|---|---|---|
| | Pregnant (n=48) | Not pregnant (n=14) | Pregnant (n=22) | Not pregnant (n=13) |
| USG | 1.033±0.001* | 1.030 (1.014-1.032) | 1.028±0.003 | 1.027±0.003 |
| pH | 8.8±0.1 | 8.6±0.4 | 8.6±0.1 | 8.5 (8.5-8.7) |
| uTP (mg/dL) | 18.8±2.1* | 23.0 (20.1- 42.4) | 13.7±1.9 | 13.1±2.0 |
| uCrea (mg/dL) | 175.5±26.5 | 169.5±34.9 | 113.0±44.5 | 81.7±24.5 |
| UPC | 0.11±0.01* | 0.15±0.02 | 0.13±0.04 | 0.17±0.05 |

N = number of specimens; n = number of urine samples; USG = urine specific gravity; uTP = urine total proteins; uCrea = urine creatinine; UPC = urine protein to urine creatinine ratio. *$P$<0.01 for the comparison between pregnant and not pregnant animals.

**Table 4:** SDS-PAGE electrophoresis results. Data are reported as mean±standard deviation

| Variable | Cows (N=5; n=29) | Heifers (N=6; n=55) | $P$* |
|---|---|---|---|
| Band Number | 14±3 | 17±3 | 0.098 |
| Band with MW > 90 kDa | 1±1 | 2±1 | 0.8 |
| Band with MW 90-14 kDa | 12±3 | 15±3 | 0.08 |

N = number of specimens; n = number of urine samples; MW = Molecular Weight. * $P$ refers to the comparison between cows and heifers.

Considering the comparison between cows and heifers, significant differences were found for uTP (P=0.0005) and uCrea (P=0.0008) (Fig. 2). Considering the pregnancy, significant differences were determined between pregnant and not pregnant heifers for uTP (P=0.009) and UPC (P=0.008) (Fig. 3). No significant differences were found between pregnant and not pregnant cows. Complete data are reported in Table 3.

**SDS-PAGE:** Representative gel and pherograms of urine samples are reported in Fig. 4. The majority of urine samples analysed in this study showed common protein bands with molecular weight (MW) of 97, 86, 76, 70, 58, 38, 27, 23, 21 kDa and less than 13 kDa. Complete data are reported in Table 4.

*Heifers* - Fifty-five samples from six heifers were analysed and a mean of 17±3 protein bands was detected. The majority of the samples presented the protein bands with molecular weight (MW) of 97, 86, 76, 70, 59, 37, 27, 23, 21, 17, 14, 13, 12 kDa.

After the first month of pregnancy, a thin band with a MW of 67 kDa appeared; this band remained visible for the entire gestation with an increase at the end of the pregnancy. From the second trimester, two bands appeared with a MW of 41 kDa and 33 kDa. Urine samples collected during the final stage of the pregnancy presented a number of protein bands higher than that determined at the initial stages.

*Cows* - Twenty-nine urine samples were analysed from five cows and a mean of 13±3 protein bands were recorded. The majority of the samples presented a common profile characterized by protein bands at MW of 97, 86, 78, 70, 67, 59, 62 and 58 kDa.

In the final stages of the pregnancy, some differences were detected: urinary proteins with MW less than 9 kDa were increased in number, while the band with MW of 11 kDa increased in intensity as gestation progresses. Moreover, urinary proteins with MW of 18 and 27 kDa appeared at the last month of the pregnancy. As reported for the heifers, the band with MW of 67 kDa was visible during the entire gestation and in the post-partum period.

Urine samples collected in the oestrus time presented a urinary proteome similar to the pregnant animals. Indeed, the bands with MW of 97, 86, 78, 70, 67, 62, 59, 58, 41, 33 and 18 kDa were present. Moreover, two additional bands with MW of 15 and 16 kDa were detected. Conversely, in these samples, the band with MW of 11 kDa was absent.

In not pregnant animals, the urinary proteome was mainly characterised by the following bands: 86, 78, 70, 59, 38, 27, 22, 11 and 9 kDa. The band at 97 kDa was scarcely evident, while the bands at 67 and 13 kDa were not present.

## DISCUSSION

Considering the importance of cattle as farm animals, previous studies were focused on the productive aspects and the quality of meat and milk as well as the strategy for their improvement (Toral *et al.*, 2018). Urinalysis was used for the evaluation of urinary nitrogen and the diagnosis of ketosis as well as for the detection of biomarkers of pregnancy and parturition (Nennich *et al.*, 2006; McArt *et al.*, 2012; Rawat *et al.*, 2012; Spek *et al.*, 2012). Despite the non-invasive sampling and the affordable price, urinalysis is still rarely used as a routine clinical diagnostic tool in farm animals. For this reason, this study investigated

the urine chemistry and the urinary proteome to obtain preliminary data on cows and heifers, to compare the electrophoretic profiles of pregnant and not pregnant animals and to evaluate their changes during the pregnancy.

**Urinalysis and UPC:** The USG value determined in this study was similar to those reported in the literature for adult cows (Herman *et al.*, 2019). In our study, the USG value was higher in heifers than in cows as well as in pregnant than in not pregnant heifers. These results could be explained considering the possible correlation between the USG and the creatinine urinary concentration, which was also higher in heifers. Indeed, the creatinine concentration is able to affect the USG value, as previously reported (Herman *et al.*, 2019). Hermann *et al.* (2019) found a strong positive correlation between the USG and the urinary creatinine concentration in beef cows. Accordingly, in our study, heifers showed a urinary creatinine concentration almost double than cattle, confirming the results reported in the literature (Herman *et al.*, 2019). The creatinine excretion through the milk could have also contributed to the differences between cows and heifers. Indeed, the cows were in lactation, while the heifers, being at their first gestation, still had to start producing milk.

Cattle urine samples had an alkaline pH, between 8 and 9, confirming the results previously reported by other authors (Herman *et al.*, 2019; Ihedioha *et al.*, 2019).

The results of the dipstick test were negative for almost all of the variables with few exceptions. In particular, the dipstick positivity to proteins should be considered with attention. The alkaline urinary pH of the cattle could have determined false positive results; therefore, quantitative measurement of proteinuria is recommended (Herman *et al.*, 2019). However, traces of proteins in bovine urine were previously reported in the literature (Mokbul *et al.*, 2016; Herman *et al.*, 2019; Ihedioha *et al.*, 2019).

Special attention should be paid also to the positivity to ketones, which was recorded in three urine samples in the post-partum period. These findings are in accordance with data previously reported in the literature for pregnant cows (Mokbul *et al.*, 2016; Ihedioha *et al.*, 2019). A metabolic switch of glucose metabolism for lactose synthesis and milk production may cause the presence of ketones in the urine (McArt *et al.*, 2012; Ihedioha *et al.*, 2019). However, the positivity determined in our study might also be originated from ruminal ketone bodies as suggested by Herman *et al.* (2019).

Regarding sediment analysis, crystals in urines, especially calcium phosphate ones, were previously reported in bovine, as well as casts and leucocytes (Mokbul *et al.*, 2016). The presence of crystals should be considered with attention and urine samples monitored to avoid obstructive urolithiasis. Indeed, in ruminants, the formation of uroliths is related to several factors, including high concentrate ration and plant rich in oxalate and phytoestrogen (Thakur *et al.*, 2019). Although crystals seemed to be frequent in urine, obstructive urolithiasis should be prevented by balancing the feeding and encouraging the animals to drink water (Parrah *et al.*, 2010).

In this study, the mean urine total protein concentrations of 13.7±1.6 mg/dL determined in cows and 20.8±2.6 mg/dL in heifers fall within the reference interval reported by Herman *et al.* (2019) for healthy cows; therefore, the animals included in our study should be considered as non-proteinuric (Herman *et al.*, 2019). Comparing the cows with the heifers, the uTP value was significantly different between the two groups ($P$=0.0005). According to data reported in the literature, the lactation number influences the UPC values, which was higher in cows during the lactation than in dry animals (Herman *et al.*, 2019). However, in our study, only the uTP value was statistically different between cows and heifers suggesting that further studies are required to investigate the changes in urine total proteins as well as in the UPC value.

**SDS-PAGE electrophoresis:** In the samples examined, common bands with apparent MW of 97, 86, 78, 70, 59, 38, 27, 23, 21 kDa and between 13 and 9 kDa were separated. The identity of some of the bands can be hypothesised basing on the apparent MW and comparing the data with those previously reported in humans and animals (Pyo *et al.*, 2003; Serafini-Cessi *et al.*, 2003; Ozgo *et al.*, 2009; Candiano *et al.*, 2010; Alhaider *et al.*, 2012; Balhara *et al.*, 2013; Brandt *et al.*, 2014; Miller *et al.*, 2014; Bathla *et al.*, 2015; Ferlizza *et al.*, 2015; Rawat *et al.*, 2016). The proteins could be identified as uromodulin (band with MW of 97 kDa), transferrin (band with MW of 78 kDa), albumin (band with MW of 70 kDa), heavy and light chains of immunoglobulins (band with MW of 59 and 27 kDa, respectively). Additionally, other proteins can be hypothesised as haptoglobin (band with MW about 38 kDa), retinol binding protein (RBP) and/or bPAP (bovine pregnancy-associated protein) (band with MW of 21-22 kDa), lysozyme and β2-microglobuline (band with MW of 13 and 9 kDa, respectively).

Uromodulin (Tamm-Horsfall protein), one of the most abundant urinary proteins in healthy mammals, was described in the urine of camel, dog, cat and humans (Serafini-Cessi *et al.*, 2003; Brandt *et al.*, 2014; Miller *et al.*, 2014; Ferlizza *et al.*, 2015). It is the main tubular protein of canine urine (Miller *et al.*, 2014) and it has also been reported in cows, where elevated concentration of uromodulin has been suggested as a pregnancy biomarker (Bathla *et al.*, 2015).

In our study, the band with MW of 78 kDa could be hypothetically identified as transferrin, which appeared as a very thin band without variation between cows and heifers and pregnant and not pregnant animals. This protein was reported also in dog urine (Brandt *et al.*, 2014). Albumin was described in many species, including humans, dog, cattle, cat, goats and camels (Pyo *et al.*, 2003; Ozgo *et al.*, 2009; Candiano *et al.*, 2010; Alhaider *et al.*, 2012; Brandt *et al.*, 2014; Miller *et al.*, 2014). In our study, the band with MW of 70 kDa might be identified as albumin, which was presented as an evident band in all the urine samples analysed.

Haptoglobin and RBP were described in feline urine and in urine of pregnant cows (Ferlizza *et al.*, 2015; Rawat *et al.*, 2016). Haptoglobin originates from plasma and it is considered a common component of healthy humans' urine, but it was also detected in urine of pregnant cows and it is a glycoprotein expressed in uterine epithelium during implantation period and increases significantly in the early days of gestation (Candiano *et al.*, 2010; Rawat *et al.*, 2016). The bovine pregnancy-associated protein (bPAP), with MW of 21 kDa, is a protein detected in the urines of pregnant cows (Pyo *et al.*, 2003).

The comparison between pregnant and not pregnant specimens showed qualitative differences, with the appearance of three bands with MW of 67, 41 and 33 kDa, which were absent in not pregnant animals. In particular, the band with MW of 67 kDa was present in all the urine samples of pregnant cows and heifers. This band might correspond to the alpha-fetoprotein (AFP), a protein produced by foetal tissues by a family of genes phylogenetically related to serum albumin (Mizejewski, 2004). The bands at 67 and 41 kDa might also be the different isoforms of Pregnancy Associated Glycoproteins (PAGs) reported in milk and serum of pregnant cows by Balhara *et al.* (2013) and suggested as useful biomarkers for early pregnancy diagnosis in bovine. However, more studies are required not only to confirm the presence of PAGs in bovine urines, but also to increase the knowledge about bovine pregnant proteome.

**Conclusion:** Urinalysis is a simple and ~~a~~ non-invasive diagnostic protocol, which should be routinely used for the clinical evaluation of large animals. The data reported in the present study could be considered suggestive of healthy animals and they confirmed those previously reported in the literature. Proteinuria should be also investigated by quantitative methods, to exclude dipstick false positive. SDS-PAGE can be considered a useful tool to study the renal function and it might be proposed for the identification of pregnancy biomarkers. However, further studies are required to improve knowledge about the bovine urinary proteome.

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**Authors contribution:** GI and GA conceived and designed the study. EF and SF executed the experiments and analysed the samples. GI, EF, SF, DC, MB interpreted the data. SF and EF drafted the manuscript. All authors critically revised the manuscript for important intellectual contents and approved the final version.

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