Effect of Slow-Release Non-Protein Nitrogen Produced from Agro-Industrial Byproducts on Bio Gas Production, Feed Digestibility and Inoculum Parameters

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**Abstract**

In the recent decades, air and surface water pollution by nitrogen from agro-industrial discards has become a global environmental concern. Generally, these byproducts and or discards are nutrient rich and could inexpensively be utilized for various purposes marginally helping with mitigation strategies. In this context, our study was conducted in two phases: producing lactosylurea from agro-industrial by-products and subsequently evaluating the possibility of using lactosylurea as a non-protein nitrogen source in the ruminant’s diet and its effect on feed digestibility as well as ruminal parameters. Gas production test and in-vitro disappearance method were used to describe the kinetics of digestion in both dry matter (DM) and crude protein (CP) of the four experimental treatments. Protozoa count and total volatile fatty acids concentration were utilized to evaluate the ruminal parameters. The treatments were 1) basal diet + urea (BDU), 2) basal diet+ lactosylurea (BDL), 3) basal diet+ concentrated lactosylurea (BDCL), 4) basal diet+ Optigen (slow release NPN) (BDO). According to our findings, produced gas, DM, and CP disappearance in were significantly higher in concentrated lactosylurea and positive control groups than the other treatments (P<0.05). Moreover, estimated metabolizable energy, digestible organic matter and short chain fatty Acid were significantly higher for the same treatments (P<0.05). The values for protozoa count (2.66×10^6 organism/ml) and total volatile fatty acids concentration (30.96 mmol/L) were significantly lower and for urea treatment compared with others (P<0.05). In conclusion, lactosylurea as agro-industrial by-products can be a good alternative for urea or Optigen to reduce environmental contamination.

**Highlights**

- Agro-industrial by-products are threatening the human and environment
- Producing new feedstuff and bio recycling can reduce pollution
- Produced slow release non-protein nitrogen and its performance was evaluated
- Slow-released urea produced from concentrated whey and urea can be used as non-protein nitrogen source
- Preserving environment by re-producing and bio recycling agro-industrial discards is possible

**Novelty**

Agro-industrial by-products are threatening the human and environment therefor producing new feedstuff and bio recycling can reduce pollution, bacteria taking part in fermentation need carbohydrate and protein to propagate their population, produced slow release non-protein nitrogen from agro-industrial discards may compensate for protein source while Preserving environment. slow release non-protein nitrogen can affect biogas production and biomass consortium.

1. Introduction
In the recent years, nitrogen utilization efficiency has been one of the challenging environmental issues. The agricultural sector contributes to the issue by adding nitrogen to the surface waters and the atmosphere (Pavlidis and Tsihrintzis, 2018; Wanapat, 2009). The ruminant’s production system should be developed to protect nature and reduce environmental pollution (Hermansen, 2003). The use of food processing by-products and discards has played a major role in the sustainability of livestock industry yet fermentation properties of these fermentable materials must be carefully investigated for optimum efficiency (VandeHaar and St-Pierre, 2006). However, it requires enough of the (Fessenden and Van Amburgh, 2016). Of all the process discards, lactose and protein rich whey is considered one of the major ones in the cheese production (Bacenetti et al., 2018). Nonetheless, not only that whey powder production is highly expensive, but the process could also pollute the environment. Whey can be used in livestock feeding as an accessible source of carbohydrates of which lactose is the most abundant one (Rocha and Guerra, 2020). On the one hand, whey as an excellent source of soluble carbohydrate for rumen microorganism, is easily fermented. On the other hand, it contains high-quality soluble protein, which could be a source of nitrogen for rumen microorganism (GUEDES et al., 2020; Lee et al., 2019).

In the cheese production industry, approximately 88% of milk remains as whey and only about 12% is converted into cheese. Utilization of whey as animal feed would reduce the environmental pollution while supplying animal feed ingredients (Ahmadi et al., 2018; Rocha and Guerra, 2020). Accordingly, providing low-cost feed is vital for a sustainable farming system and whey can be a suitable inexpensive substitute for livestock feed. Feed protein is one of the most expensive and restrictive part of livestock feed (Kim et al., 2019). Non-protein nitrogen (NPN) sources are good alternatives for diet protein due to their lower cost compared to true protein (Calsamiglia et al., 2010; Cherdthong and Wanapat, 2010; Taylor-Edwards et al., 2009). Ruminants can convert NPN to milk protein (Virtanen, 1966) where using urea as NPN source could supply adequate nitrogen for rumen microbial growth and amino acid flow into small intestine (Fessenden et al., 2019). The issue of rapid release of ammonia, when rumen fluid urease reacts with the animal consumed urea, seems an important limitation in the efficient utilization of urea. Generally, hydrolysis rate of ammonia is faster than that of ammonia used by ruminal bacteria, leading to the waste of molecule (Galo et al., 2003; Inostroza et al., 2010). However, controlled release rate of urea simultaneous with the carbohydrate breakdown could be a solution to the issue (Taylor-Edwards et al., 2009). Slow-release urea products are a good alternative to ruminant’s diet protein (Joysowal et al., 2019; Kertz, 2010). Lactosylurea is a combination of urea and whey for the simultaneous availability of nitrogen and carbohydrates (Álvarez-Cao et al., 2020). In this context, the main objective of this study was to investigate the use of whey-derived lactosylurea and urea as agro-industrial by-products on feed digestibility and ruminal parameters. The specific objective of our study was to reduce the environmental contamination by secondary by-products of human-food producing industry.

2. Materials And Methods

2.1. Samples preparation
Lactosylurea samples were prepared in two different conditions as first and second methods with slight differences according to the methods proposed by Merry et al. (1982) and Torkashvand and Nezamedost (2009). In the first method, 200 ml of whey (provided by Pegah Factory of Tabriz, East Azarbaijan, Iran.) was mixed with 0.11 ml of sulfuric acid and 0.125 g of urea and incubated in 55°C for 72 h. Neutralization was performed with sodium hydroxide and centrifuged for 10 min at 1300 rpm. The residue was separated, and the remaining liquid was transferred to the refrigerator. After the formation of white crystals, they were separated from the initial liquor and stored at 37°C for 24 h in a vacuum drying oven. In the second method, 200 ml of concentrated whey (provided by Pegah Factory of Tabriz, East Azarbaijan, Iran) was mixed with 0.22 ml of sulfuric acid and 0.25 g of urea and incubated at 55°C for 48 h. Then, the solution was neutralized with sodium hydroxide and centrifuged (10 min, 1300 rpm). The supernatant was transferred to the refrigerator. Then, the formed crystals were washed twice with distilled water and placed in an oven under vacuum for 24 h at 37°C.

Experimental treatments were prepared in four separate Total mixed ration (TMR) diets with varying source of NPN. The test diets were: (1) basal diet + urea (BDU), (2) basal diet + lactosylurea (first method) (BDL), (3) basal diet + concentrated lactosylurea (second method) (BDCL), (4) basal diet + Optigen (commercial slow release NPN source) (BDO)

2.2. Chemical analysis of diets

Proximate analysis of individual feedstuffs was determined based on the proposed methods of Association of Official Analytical Chemists (AOAC) dry matter (method 930.15), crude protein (method 984.13), ether extract (method 920.39) and crude ash (method 942.05), to balance TMR diets. Neutral detergent fiber and ADF were determined according to the previously described method of Van Soest et al. (1991). Test diets were formulated according to NRC (2001) requirements for cows with an average of 39 kg milk yield.

2.3. Gas production test and in-vitro digestibility

We loaded 300 mg of the ground test treatments (Wiley Mill, 2mm) into four 50 ml glass phials. Buffer solution (synthetic saliva) was prepared as proposed by McDougall (1948). Ruminal fluid of at least three freshly slaughtered beef (Lutakome et al., 2017) were percolated through a 4-layer cheese cloth to a flask which had been warmed at 39°C, and promptly taken to the laboratory. Strained ruminal inoculums were mixed thoroughly at 39°C together with the synthetic saliva (1:2 v/v) to have a homogeneous digestion medium. Glass phials were loaded by 20 ml of homogeneous digestion medium in six replicates for each treatment. Only digestion medium was loaded into blank phials. Phials were placed in shaker adjusted to 39°C and 120 rpm (Shirmohammadi et al., 2020). Gas production data were recorded at 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h of incubation (Fedorah and Hrudey, 1983; Gallo et al., 2016).

2.4. In-vitro disappearance method

In-vitro digestibility of treatments were determined according to Khajehdizaj et al. (2014). Briefly, along with gas production test, five vials of loaded treatments with digestion medium were incubated for 2, 12,
24 and 48 h, but in order to release the vials gas production during incubation hours syringe needles were fitted to the sealed vials cap. After each incubation hour vials were stored at -20°C till further analyses. Prior to analyses, vials were thawed at 39°C and contents were transferred to 50 ml falcon tubes. Subsequently, tubes were centrifuged (3000 g, 10 min), supernatant was pipetted out and the pellets were rinsed three times with phosphate buffer (pH 7.4) (Wang et al., 2012). Sample residuals were oven dried (60°C), weighed and analyzed for CP content. Consequently, CP digestibility calculated according to the following equation.

\[
\text{in-vitro CP digestibility} = \frac{\text{[[DM in the sample]*[CP of sample]]}-\text{[[DM in the residue]*[CP of residue]]}}{\text{[[DM in the sample]*[CP of sample]]}} \times 100
\]

### 2.5. Ruminal parameters

#### 2.5.1. Protozoa count

Ruminal fluid samples, collected from the slaughterhouse, were added to experimental diet containing vials (triplicates) and incubated for 12 h. Samples were withdrawn from the incubator and the protozoa were fixed using formaldehyde solution (1:4). The number of protozoa was counted according to the method of Dehority et al. (1989).

#### 2.5.2. Measurement of total volatile fatty acids

Total volatile fatty acid content of the samples was measured according to a previously described method Markham (1942). Briefly, total volatile fatty acids of rumen fluid that incubated in three replicates for 12 h were measured in two distillation and titration steps. After collection, about 50 ml of the solution in distillation step, it was titrated by addition of a few drops of phenolphthalein reagent with 0.05 N NaOH solution.

### 2.6. Calculations and Statistical model

All documented data were analyzed in a complete randomized design (CRD) utilizing SAS software (version 9.2, the ANOVA procedure, Duncan's multiple range test). Gas production kinetic was calculated by the following model:

\[
y = A \left(1 - e^{-ct}\right)
\]

Where \(A\) is the gas production from the immediately soluble and insoluble fraction; \(c\) is the rate constant (\%/h) of gas production from the insoluble fraction; \(t\) is the incubation time (h); and \(Y\) is the volume of gas produced at time \(t\).

The Metabolizable Energy (ME) (MJ/kg DM) was calculated with the equations assayed by Getachew et al. (2002), and Digestible Organic Matter (DOM) also Short Chain Fatty Acid (SCFA) were estimated according to Menke et al. (1979).

\[
\text{ME (MJ/kg DM)} = 1.06 + 0.157 \text{ GP} + 0.084 \text{ CP} + 0.220 \text{ CF} - 0.081 \text{ CA}
\]
DOM (% DM) = 9.00 + 0.9991 GP + 0.0595 CP + 0.0181 CA

SCFA (m mol/200 mg DM) = 0.0222GP − 0.00425

3. Results And Discussion

Experimental diets compositions are shown in Table 1. Experimental diets, except for NPN source, had same composition and were assumed to be fed at the dosage of 60 gram per cow per day at the farm level.

3.1 Gas production

The obtained data from the gas production (mL/g DM) of the experimental diets were tabulated in Table 2. According to the obtained results, BDCL had the highest gas production volume (290.91 mL/g DM) after 96 h of incubation which was considerably different from BDL diet. The lowest volume of produced gas was related to BDU. We found no significant differences between treatments at the initial incubation times (P<0.05), but after 6 h of incubation significant differences were observed (P<0.05). BDL and BDCL did not differ significantly up to 48 h, which could be due to their same NPN source. BDU showed a significant difference with the treatments containing Lactosylurea and Optigen, which might be due to the faster release of ammonia from urea compared with the other NPN sources after 4 h of incubation. BDU had the lowest gas production at all incubation times except for the first 4 h (P<0.05). In the present study, we estimated higher extent of fermentation (A (ml/g DM)) for BDCL and BDO, however, BDCL had lower rate (c ml/h) of gas production (P<0.05). Ruminal bacteria consortium is able to produce biogas from different sources of feedstuff (Zhang et al., 2016). Cherdthong and Wanapat (2010) reported the highest gas production for urea calcium (product of slow-release urea) along with cassava chips. They reported that an increase in the extent and rate of fermentation was related to the energy source (cassava chips). The cause of high gas production for BDCL was probably related to the presence of lactose as energy source in concentrated lactosylurea. The high cumulative amount of produced gas indicates high metabolic energy, fermentable nitrogen and other nutrients for the activity of microorganisms Menke et al. (1979). Gas production is positively correlated with the dry matter digestibility indicating that gas production is an integral part of food fermentation. Usually, high gas production was achieved from the carbohydrate section of the feed compared to the other nutrients. The amount of gas production in the early times is due to the differences in the level of nonstructural carbohydrates (NSC) such as sugars, pectin, and starches that are rapidly fermenting and producing gas (Menke, 1988), yet, we didn't observe any significant differences at early hours (P<0.05).

Calculated gas production parameters including ME, NE\textsubscript{l}, DOM, SCFA are reported in Table 3.

BDCL and BDO had the highest values in the calculated parameters (P<0.05). BDU had obtained the lowest value for all parameters. BDL showed significant difference with the rest of the treatments (P<0.05). In a recent study conducted by Besharati et al. (2019) on the effect of adding whey and L.
Buchneri to alfalfa silage on in vitro gas production and degradability, they reported that adding fresh whey had increased Calculated gas production parameters.

### 3.2 The apparent degradability of dry matter

The mean data for dry matter disappearance are presented in Table 4. According to the results, BDCL and BDO showed the highest degradabilities after 12 h of incubation (P<0.05). Less than 12 h incubation, treatments had no significant difference (P<0.05). After 24 h there were significant differences not only with BDU but also with BDL. The later difference may be caused by the amount of nutrients presented in the whey as the primary substance in lactosylurea production. Chamebon et al. (2017) observed that dry matter degradability increased by adding urea to the treatments (to orange pomace treatment with 38.5% of wheat straw and 1.5% urea and orange pomace treatment with 37% of wheat straw and 3% urea) compared with non-urea treatment. Mahmoudi-Abyane et al. (2018) studied the effect of utilizing different sources of nitrogen on digestibility and nitrogen balance in Mehraban lambs. They reported the lowest and highest digestibility of NDF and ADF for diet containing soybean meal and slow-release urea, respectively. These results suggested that diets with a contentious source of nitrogen can meet the requirements of the cellulolytic bacteria, the major ammonia consumers in the rumen, consequently improving fiber digestion and the activity of rumen microorganisms (Castro et al., 1999). Ruminal bacteria can receive 40 to 95% of their nitrogen from ammonia depending on the diet, and using these sources can create a balance of peptides and amino acids (Nolan et al., 1993). In a previous study, digestibility of the fibers increased when Optigen was replaced with soybean meal and rapeseed meal, although ammonia nitrogen was high in both treatments (Sinclair et al., 2012). It has been reported that adding 1.8 kg slow-release urea supplementation to the beef diet containing sugarcane, cane molasses and maize significantly improved the digestibility of dry matter and NDF (Galina et al., 2003).

### 3.3 The apparent degradability of crude protein

The mean data of crude protein degradability of the experimental treatments are presented in Table 4. According to the obtained results, the highest degradability was observed in BDCL and BDO. The lowest degradability was reported in BDU. Treatments showed no significant differences at 2 h but differences were significant after 12 h (P<0.05). This difference may be due to the presence of lactose in BDCL and BDO, which showed nitrogen release compared with other two treatments and Optigen as a commercial slow-release urea product had the kinetics just like BDCL. A recent report by Sevim and Önl (2019) that worked on the effect of supplemental slow-release urea on some feed digestibility and rumen parameters showed that using slow release urea had increased feed protein digestibility which is in line with ours.

### 3.4 Protozoa count

The results of the number of protozoa are presented in Table 5. The number of protozoa in the BDCL and BDL treatments were significantly higher than the other treatments (P<0.05). The absence of protozoa reduces the predation of bacteria (Takahashi et al., 2005) resulting reduced end products from ruminal bacteria degradation while increasing the flow of microbial protein into the lower gastro intestinal tract.
(Hess et al., 2004). The protozoa can use up starch granules, thereby creating a balance in the rumen environment and better cellulose digestion (Orpin, 1984). Eugène et al. (2004) found that use of high levels of concentrate can reduce the protozoa population due to a reduction in ruminal pH. In the present study, diets contained equal concentrate: forage ratio and the only source of difference was related to NPN source, therefore the difference in the number of protozoa cannot be related to the mentioned fact. Protozoa use cellulose and starch as a source of energy, and ruminal bacteria and insoluble proteins as a source of nitrogen (Coleman, 1986; Jouany, 1996). Diets with high concentrate provide digestible energy sources for the protozoa boosting their growth yet allowing protozoa to better compete with ruminal bacteria (Yuste et al., 2019). According to the results of this study, it can be expressed that the diets containing lactosylurea and Optigen can provide nitrogen source easily available for both of ruminal bacteria and protozoa.

3.5 Ruminal total volatile fatty acids

The results of ruminal total VFA (mmol/ml of rumen fluid) after 12 h of incubation are presented in Table 6. The lower concentration of volatile fatty acids was observed in the BDU and the higher was recorded for the others (P<0.05). It can be explained that in the treatments containing slow-release urea source, the levels of ruminal VFA significantly were high in comparison with the other NPN sources (P<0.05). This case might be related to the final products of ruminal microbial protein in the rumen fluid. Therefore, more microbial protein synthesis that probably occurred in three treatments containing of slow-release urea sources in comparison with diet containing of unprocessed urea (BDU). The ruminal total VFA concentration can varied widely depending on the diet variance and elapsed time from the previous meal (Jeong et al., 2016). Taylor-Edwards et al. (2009) tested 1.6% of urea on calves and reported VFA at 99.7 (mmol/ml), whereas in our study treatment containing 0.26% of urea in the TMR diet was reported at 30 (mmol/ml). The reason for this difference could be related to variance in urea levels and the incubation time. The same archer estimated the level of VFA for the treatment containing of 1.6% urea (103.2 mmol/ml), which is in consistent with the results of our studies (BDCL and BDO). Pinos-Rodríguez et al. (2010) estimated ruminal total VFA in treatments containing 0.6% and 1.1% of Optigen at 97.6 and 94.8 mmol/L, respectively. The higher percent of Optigen reduced this parameter and it agreed with the obtained data from the diet containing Optigen (BDO). In an experiment on dairy cows, Xin et al. (2010) did not show a difference between urea and the coated urea product in terms of total VFA, which contrasted with the results of this study.

Conclusion

According to our observations, lactosylurea, synthesized using urea and whey, can be a suitable alternative for urea or Optigen since in-vitro dry matter and crude protein disappearance values as well as gas production was improved in the lactosylurea containing diets. Additionally, it could significantly improve selected rumen parameters inclusive of ruminal protozoa count and total VFA concentrations. Therefor it is suitable to make use of it to proceed sustainable dairy farming beside preserving environment.
Declarations

Acknowledgment

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Declaration of interests

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.

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Tables

Table 1. Ingredient and chemical composition of the experimental diets (%DM)
| Item                              | Diet  |
|----------------------------------|-------|
|                                  | BDU   | BDL  | BDCL | BDO  |
| Ingredients, % of DM             |       |      |      |      |
| Corn silage                      | 20.07 | 20.07| 20.07| 20.07|
| Alfalfa hay                      | 18.24 | 18.24| 18.24| 18.24|
| Wheat straw                      | 0.73  | 0.73 | 0.73 | 0.73 |
| Cottonseed, whole                | 7.36  | 7.36 | 7.36 | 7.36 |
| Beet sugar pulp                  | 1.87  | 1.87 | 1.87 | 1.87 |
| Barley grain, ground             | 15.04 | 15.04| 15.04| 15.04|
| Corn grain, ground               | 14.63 | 14.63| 14.63| 14.63|
| Corn gluten meal                 | 4.12  | 4.12 | 4.12 | 4.12 |
| Soybean meal (solvent extracted) | 6.17  | 6.17 | 6.17 | 6.17 |
| Soybean seeds, whole, heated     | 3.43  | 3.43 | 3.43 | 3.43 |
| Sunflower meal, solvent          | 0.91  | 0.91 | 0.91 | 0.91 |
| Meat and bone, rendered          | 2.33  | 2.33 | 2.33 | 2.33 |
| Canola meal, mesh. Extract       | 1.42  | 1.42 | 1.42 | 1.42 |
| Corn germ                        | 0.73  | 0.73 | 0.73 | 0.73 |
| Wheat bran                       | 0.32  | 0.32 | 0.32 | 0.32 |
| Calcium soap of fatty acids      | 0.5   | 0.5  | 0.5  | 0.5  |
| Salt                             | 0.18  | 0.18 | 0.18 | 0.18 |
| Calcium phosphate (Di)           | 0.41  | 0.41 | 0.41 | 0.41 |
| Calcium carbonate                | 0.41  | 0.41 | 0.41 | 0.41 |
| Magnesium oxide                  | 0.09  | 0.09 | 0.09 | 0.09 |
| Sodium bicarbonate               | 0.5   | 0.5  | 0.5  | 0.5  |
| Vitamin & Mineral premix         | 0.5   | 0.5  | 0.5  | 0.5  |
| Urea g/d/cow                     | 60    | -    | -    | -    |
| Lactosylurea g/d/cow             | -     | 60   | -    | -    |
| Concentrated Lactosylurea g/d/cow| -     | -    | 60   | -    |
| Optigen g/d/cow                  | -     | -    | -    | 60   |
| Composition, % of DM |
|---------------------|
| CP                  | 17 | 17 | 17 | 17 |
| RDP                 | 10.7 | 10.7 | 10.7 | 10.7 |
| RUP                 | 6.3 | 6.3 | 6.3 | 6.3 |
| NDF                 | 31.1 | 31.1 | 31.1 | 31.1 |
| ADF                 | 23.1 | 23.1 | 23.1 | 23.1 |
| NFC                 | 41.9 | 41.9 | 41.9 | 41.9 |
| Ca                  | 1 | 1 | 1 | 1 |
| P                   | 0.6 | 0.6 | 0.6 | 0.6 |
| NEL, Mcal/kg        | 1.66 | 1.66 | 1.66 | 1.66 |
| Lys/Met g/d         | 3.12 | 3.12 | 3.12 | 3.12 |

BDU (basal diet + urea), BDL (basal diet+ lactosylurea (first method)), BDCL (basal diet+ concentrated lactosylurea (second method)), BDO (basal diet+ Optigen (commercial slow release NPN source))

Corn silage was 28.98% DM and (DM basis): 48.97% NDF and 6.84% CP.

Alfalfa hay contained (DM basis): 42.70% NDF and 14.17% CP.

Wheat straw contained (DM basis): 56.77% NDF and 2.9% CP.

Cottonseed, whole contained (DM basis): 74.5% NDF and 7.9% CP.

Beet sugar pulp contained (DM basis): 37.48% NDF and 7.97% CP.

Barley grain, ground contained (DM basis): 15.53% NDF and 11.51% CP.

Corn grain, ground contained (DM basis): 12.32% NDF and 8.57% CP.

Corn gluten meal contained (DM basis): 31.33% NDF and 58.85% CP.

Soybean meal (solvent extracted) contained (DM basis): 14.85% NDF and 44.88% CP.

Soybean seeds, whole, heated contained (DM basis): 21.83% NDF and 34.83% CP.

Sunflower meal, solvent (DM basis): 35.83% NDF and 29.72% CP.

Meat and bone, rendered (DM basis): 49.62% CP.
Canola meal, mesh. Extract (DM basis): 31.53% NDF and 30.11% CP.

Corn germ (DM basis): 53.07% NDF and 18.23% CP.

Wheat bran contained (DM basis): 31.86% NDF and 14.43% CP.

### Table 2. Cumulative gas production of treatment samples (ml/g of DM) (p<0.05).

| incubation hour | experimental diets |          |          |          | SEM  | P value |
|-----------------|--------------------|----------|----------|----------|------|---------|
|                 | BDU                | BDL      | BDCL     | BDO      |      |         |
| 2               | 29.56              | 26.49    | 26.76    | 29.56    | 0.92 | 0.074   |
| 4               | 62.86              | 64.12    | 63.86    | 65.52    | 1.37 | 0.664   |
| 6               | 86.75<sup>b</sup>  | 94.22<sup>a</sup> | 94.35<sup>a</sup> | 95.42<sup>a</sup> | 1.94 | 0.039   |
| 8               | 116.68<sup>b</sup> | 127.48<sup>a</sup> | 127.68<sup>a</sup> | 128.42<sup>a</sup> | 2.16 | 0.008   |
| 12              | 170.02<sup>b</sup>| 186.62<sup>a</sup> | 190.68<sup>a</sup> | 189.47<sup>a</sup> | 2.16 | 0.0001  |
| 24              | 206.10<sup>b</sup>| 240.43<sup>a</sup> | 246.43<sup>a</sup> | 246.96<sup>a</sup> | 2.08 | 0.0001  |
| 48              | 232.59<sup>c</sup>| 254.46<sup>b</sup> | 268.72<sup>a</sup> | 264.46<sup>a</sup> | 2.66 | 0.0001  |
| 72              | 242.64<sup>c</sup>| 266.18<sup>b</sup> | 284.44<sup>a</sup> | 276.84<sup>a</sup> | 2.97 | 0.0001  |
| 96              | 248.98<sup>c</sup>| 273.04<sup>b</sup> | 290.91<sup>a</sup> | 283.58<sup>a</sup> | 3.10 | 0.0001  |
| A<sup>1</sup>, ml/g DM | 230.99<sup>c</sup>| 276.33<sup>b</sup> | 286.28<sup>a</sup> | 288.79<sup>a</sup> | 1.78 | 0.0001  |
| c<sup>2</sup>, h<sup>-1</sup> | 0.103<sup>a</sup> | 0.093<sup>b</sup> | 0.090<sup>b</sup> | 0.087<sup>b</sup> | 0.003 | 0.0707  |
| Lag<sup>3</sup>, h | 0.88<sup>b</sup> | 1.090<sup>a</sup> | 1.109<sup>a</sup> | 0.962<sup>b</sup> | 0.041 | 0.0202  |

Within a column, means without a common superscript letter differ (P<0.05).

BDU (basal diet + urea), BDL (basal diet+ lactosylurea (first method)), BDCL (basal diet+ concentrated lactosylurea (second method)), BDO (basal diet+ Optigen (commercial slow release NPN source))

<sup>1</sup>A = asymptotic gas production (ml/g DM incubated);

<sup>2</sup>c = fractional rate of fermentation (h<sup>-1</sup>);

<sup>3</sup>Lag = lag time (h);
Table 3: Estimated gas production parameters (p<0.05).

| Item                  | Experimental diets | BDU | BDL | BDCL | BDO | SEM | P value |
|-----------------------|--------------------|-----|-----|------|-----|-----|---------|
| ME (MJ/kg DM)         |                    | 10.54<sup>c</sup> | 11.61<sup>b</sup> | 11.80<sup>a</sup> | 11.82<sup>a</sup> | 0.025 | 0.0001  |
| NEI (Mcal/kg DM)      |                    | 4.56<sup>c</sup> | 5.35<sup>b</sup> | 55.49<sup>a</sup> | 5.50<sup>a</sup> | 0.019 | 0.0001  |
| OMD (% DM)            |                    | 51.32<sup>c</sup> | 58.11<sup>b</sup> | 59.36<sup>a</sup> | 59.45<sup>a</sup> | 0.165 | 0.0001  |
| SCFA (mmol/200mgDM)   |                    | 0.91<sup>c</sup> | 1.06<sup>b</sup> | 1.08<sup>a</sup> | 1.09<sup>a</sup> | 0.0004 | 0.0001  |

Within a raw, means without a common superscript letter differ (P<0.05).

BDU (basal diet + urea), BDL (basal diet+ lactosylurea (first method)), BDCL (basal diet+ concentrated lactosylurea (second method)), BDO (basal diet+ Optigen (commercial slow release NPN source))

Table 4: In-vitro disappearance of dry matter and crude protein (p<0.05).

| Item                  | Incubation time (h) |
|-----------------------|---------------------|
|                       |                    | 2        | 12       | 24       | 48       |

Invitro disappearance of dry matter (% DM)

| Item | 2      | 12     | 24    | 48    |
|------|--------|--------|-------|-------|
| BDU  | 21.6   | 34.6<sup>b</sup> | 44.9<sup>c</sup> | 53.0<sup>c</sup> |
| BDL  | 21.5   | 38.5<sup>a</sup> | 46.5<sup>b</sup> | 55.6<sup>b</sup> |
| BDCL | 21.8   | 38.5<sup>a</sup> | 49.8<sup>a</sup> | 59.4<sup>a</sup> |
| BDO  | 21.9   | 38.2<sup>a</sup> | 49.5<sup>a</sup> | 59.2<sup>a</sup> |
| SEM  | 0.43   | 0.38   | 0.29  | 0.46  |
| P value | 0.881 | 0.0001 | 0.0001 | 0.0001 |

Invitro disappearance of crude protein (% DM)

| Item | 2        | 12        | 24       | 48       |
|------|----------|-----------|----------|----------|
| BDU  | 13.25    | 30.33<sup>b</sup> | 38.09<sup>c</sup> | 45.85<sup>c</sup> |
| BDL  | 13.50    | 34.83<sup>a</sup> | 43.02<sup>b</sup> | 51.97<sup>b</sup> |
| BDCL | 13.85    | 34.65<sup>a</sup> | 47.41<sup>a</sup> | 55.22<sup>a</sup> |
| BDO  | 13.45    | 33.89<sup>a</sup> | 47.62<sup>a</sup> | 55.38<sup>a</sup> |
| P value | 0.927 | 0.005 | 0.0001 | 0.0001 |
| SEM  | 0.64     | 0.84      | 0.99     | 0.66     |

Within a column, means without a common superscript letter differ (P<0.05).

BDU (basal diet + urea), BDL (basal diet+ lactosylurea (first method)), BDCL (basal diet+ concentrated lactosylurea (second method)), BDO (basal diet+ Optigen (commercial slow release NPN source))
Table 5: Total volatile fatty acid and protozoa in rumen fluid (p<0.05).

| item               | Experimental diets | BDU | BDL       | BDCL      | BDO       | P value | SEM  |
|--------------------|--------------------|-----|-----------|-----------|-----------|---------|------|
| VFA (mmol/Lit)     |                    | 30.96<sup>b</sup> | 101.29<sup>a</sup> | 104.44<sup>a</sup> | 104.60<sup>a</sup> | 0.0001  | 1.18 |
| Protozoa (10<sup>6</sup> organisms/ml) | 2.66<sup>c</sup> | 4.06<sup>b</sup> | 4.66<sup>a</sup> | 4.80<sup>a</sup> | 0.0001  | 0.13 |

Within a row, means without a common superscript letter differ (P<0.05).

BDU (basal diet + urea), BDL (basal diet+ lactosylurea (first method)), BDCL (basal diet+ concentrated lactosylurea (second method)), BDO (basal diet+ Optigen (commercial slow release NPN source))

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Table 6 is not available with this version