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Effect of Feeding Lucerne and a Mixed Diet of Oats and Berseem Clover as a Source of Fresh Forage on Ruminal Characteristics and Nitrogen Use Efficiency in Dairy Cows during the Winter Period

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Abstract: The inclusion of fresh forages into dairy cows' diets during the winter can represent economic and nutritional benefits but can affect cows' metabolic function. The study aimed to evaluate the effect of using fresh berseem clover/oat (MIX), fresh lucerne (LEG) and hay/silage (CON) as forage basis for total mixed ration during the winter period on dairy cows' rumen characteristics and N metabolism. Three non-lactating rumen-cannulated cows were allocated to each diet for a 14-day period in a 3 × 3 Latin Square design. Sample collection occurred on the last 3 days of each period. Rumen fluid samples were analysed for pH, volatile fatty acids and N-ammonium contents. In situ forage nutrients degradability were evaluated by ruminal incubation. Serum, urine and faecal samples were collected and analysed for N content. Cows had similar feed intakes. No major changes were observed in rumen characteristics, but LEG led to greater ruminal N-ammonium and ammonium ureic *N* contents. Fresh pasture diets reduced the daily urine ureic N. The fresh forages improved the ruminal kinetics of dry matter and crude protein. The forage nutrients' effective degradability was greater for the fresh pasture diets than for CON. Overall, inclusion of fresh forages had minor effects on ruminal parameters, but the use of the MIX diet represents a suitable option in terms of N use efficiency.

Keywords: *Trifolium alexandrinum*; legumes fermentation; rumen degradability; nitrogen utilization; nutrient degradability; volatile fatty acids; forage utilization; cattle nutrition; zero-grazing

1. Introduction

During winter, in areas with Mediterranean climate such as central Chile, the Mediterranean basin, California and Australia, pasture production and forage supply is low due to low temperatures and shorter daylight [1]. Under these conditions, the pasture growth rate and nutritional value does not meet lactating dairy cows' nutritional requirements for maintenance and milk production, and thus, total mixed rations (TMR) based on concentrates and preserved forages such as hay or silage or partial mixed rations where fresh forages can be partially included are commonly offered.

During the forage conservation process, there are losses of dry matter (DM) [2] and a reduction in the concentration of nutrients as a result of physical and chemical processes that decrease the nutritional value of hay and silage [3,4]. Proteins and water-soluble carbohydrates (WSC) are among the nutrients that decrease during the conservation process of fresh forages [5,6]. The decrease in the content and availability of these nutrients is the reason why the nutritional value of the preserved forage is commonly lower than that of the original forage [7].

Inclusion of fresh forages into a TMR ration reduces costs associated to forage conservation and storage [8], reduces nutrient losses and increase the proportion of certain nutrients in milk (e.g., rumenic and vaccenic fatty acids, FA) that can have a positive effect on human health [9]. Avoiding the degradation of proteins and WSC that affects forages during the conservation process could directly influence nutrients metabolism at the ruminal level, altering passage rate, volatile fatty acids (VFA) and nutrients digestibility [10].

Several authors have studied the effect of including fresh forage in ruminant diets on nutrients digestibility and ruminal metabolism [8,11–13]. Bargo, et al. [12] reported a decrease in the content of urea in plasma and milk, and a higher percentage of milk protein in cows fed with 100% TMR diets or combined with pastures (15 kg DM of daily herbage allowance) compared to animals fed only pastures, with no differences in ruminal pH nor in the total content of VFA [13]. In housed dairy cows fed TMR diets with more than 8 h a day of access to high quality fresh Italian ryegrass (*Lolium multiflorum* L.), Mendoza, et al. [14] found a decrease in the amount of total VFA and N-NH_4^+ in the rumen and an increase in the proportion of butyric acid compared to when they were fed TMR only.

Berseem clover (*Trifolium alexandrinum* L.; BC) is an annual legume frequently used as fresh winter fodder in Mediterranean and subtropical climates [15,16] as it has a good winter growth rate and high nutritional value [17]. It is a forage crop appreciated due to its high protein content, comparable to that of lucerne (*Medicago sativa* L.; LUC) [18,19], and can even exceed the expected contribution of metabolizable protein for lactating dairy cows of most perennial legumes and perennial ryegrass during the winter period [20]. In addition, BC adapts to a wide range of environments, being able to fix more than 200 kg N/ha per year [21], and in contrast with other species of forage legumes it does not cause bloat in ruminants [17].

Some studies have evaluated the effect of including BC preserved as hay or silage in the diet of ruminant species. In an In Situ study it was observed that feeding dairy cows with BC silage resulted in lower CP degradability and greater neutral detergent fibre (NDF) degradability compared to LUC silage [22]. These effects could be related to ruminal fermentation variables such as pH, VFA and ruminal N-NH_4^+ in cows fed BC. However, buffalos fed diets based on BC hay showed similar digestibility of nutrients such as NDF, acid detergent fibre (ADF) and cellulose compared to LUC hay, as well as the total concentration of VFA, total N and N-NH_4^+ in ruminal liquor [23]. Karsli, et al. [24] also reported that the effects of supplementing BC hay on the consumption and digestibility of maize residues in sheep are similar to that of supplementing with LUC hay.

To meet requirements of dairy cattle, especially in critical periods such as autumn and winter, BC is generally used with oats (*Avena sativa* L.; OAT), either supplied as fresh forage or preserved as silage [25], since the intercropping of BC with cereals can increase the yield and nutritional value of the produced forage [26,27]. However, to the best of our knowledge, no previous studies have reported on the use of this mixture as fresh forage and on dairy cattle. Therefore, the objective of the study was to determine the effect of a mixed pasture of BC/OAT or LUC as a source of fibre and protein in a TMR, on the ruminal parameters, the degradability of nutrients In Situ and the N use efficiency (NUE) of dairy cows. We hypothesize that, when compared to conserved forages, the use of fresh forages as a source of fibre and protein in a TMR for dairy cows will (1) improve rumen fermentation parameters (pH and total VFAs) due to their (2) greater nutrients (DM, CP, NDF and ADF) degradability and thus, (3) will result in improved nitrogen use efficiency.

2. Materials and Methods

This study was carried out at the experimental station of the Pontificia Universidad Católica de Chile, located in Pirque (33°40′ S, 70°36′ W), for six weeks in the 2016 spring-winter period (August to October). The study was reviewed and approved by the Scientific Ethics Committee for Animals and Environmental Care of the Pontificia Universidad Católica de Chile (protocol number 160511004).

2.1. Animals and Treatments

Three non-pregnant, non-lactating dairy cows with ruminal cannula (no. 3C, Bar Diamond Inc., Boise, ID, USA) were used. Each cow was offered each of the diets according to a Latin Square 3 × 3 experimental design with three diets and three periods. The three diets were iso-energetic formulated and composed of forage and concentrate in a 55:45 ratio. The control diet (CON) consisted of a TMR diet with maize silage and LUC hay as fibre sources. In the two experimental diets, the fibre source was replaced by fresh forage, either BC with OAT (MIX diet) or LUC (LEG diet). The concentrate feed was the same in the three treatments (Table 1). Each period of the Latin square lasted 14 days consisting of 11 days for diet adaptation and the last 3 days for sample collection. The cows were housed in individual pens (3.0 m × 9.5 m) with access to water *ad libitum*. Each cow received 13 kg DM/d of TMR, according to the nutritional requirements of the animal during a dry period [28]. Approximately 60% of feed was offered in the morning and the remaining feed in the afternoon.

Table 1. Ingredients of the diets used in the experiment: control (CON), lucerne (LEG) and berseem clover/oat (MIX).

	Diets		
Ingredients (kg of DM)	**CON**	**LEG**	**MIX**
Lucerne hay	2.77	-	-
Maize silage	5.13	-	-
Fresh berseem clover/oat forage		-	7.90
Fresh lucerne forage		7.90	
High moisture maize grain	0.62	0.62	0.62
Crushed soybean	1.71	1.71	1.71
Wheat middling	2.77	2.77	2.77

Cuts of the LUC and BC/OAT pastures were made with an electric grass cutter (Mitsubishi, Tl33) at 08:00 h to harvest the forage for the day. Green matter not used in the morning feed was stored indoors protected from rain and direct sunshine exposure until the afternoon feed. The morning and afternoon TMR were prepared manually by mixing diets ingredients homogeneously.

2.2. Forage and Concentrate Measurements

Forage samples were taken three times a week while the concentrate was sampled once a week (500 g/sample). The samples were dried in an oven at 60 °C for 48 h to determine the DM content, were ground and sieved through a 1 mm mesh and stored for chemical analysis. The AOAC [29] methods were used to estimate DM (method 2001.12), CP (method 2001.11), ether extract (method 920.39), lignin (method 973.18) and ash (method 942.05) concentrations. The neutral detergent fibre (NDF) and acid detergent fibre (ADF) concentrations were determined using the Van Soest [30] method. The neutral detergent insoluble nitrogen (NDIN) and the acid detergent insoluble nitrogen (ADIN) fractions were determined by estimating the CP concentration (method 2001.11) of the NDF and ADF fractions, respectively. The metabolizable energy (ME) and the total digestible nutrients (TDN) were estimated according to NRC [28] equations.

To estimate the botanical composition of the pastures as the proportion of each species (DM basis), a sample of the forage was extracted from the area that was going to be cut for two days of feeding. The pasture was traversed following a W-shaped transect, and cuts were made every 10 steps (approximately 4 cm above the ground) using a hand shear (Accu 60; Gardena International GmbH, Ulm, Germany). Each sample was thoroughly mixed, and a 100 g subsample was manually separated into BC, OAT, ryegrass, and weeds for the BC/OAT forage, and in LUC and weeds for the LUC forage, and then each fraction was dried in an oven at 100 °C for 6 h to estimate the DM content.

2.3. Sampling and Analysis of Ruminal Fluid

Individual rumen fluid samples were collected in the morning (9:00 h) and afternoon (15:00 h) on days 12 and 14 of each period. The samples were taken from the cranio-dorsal, cranio-ventral, caudo-dorsal and caudo-ventral areas of the rumen using a ruminal extraction probe (RT Rumen Fluid Sampler). Rumen fluid pH was measured immediately using a pH meter (PP-201 GOnDO Electronic Co., Ltd., Taipei City, Taiwan). Two 10 mL subsamples were taken for determining $N\text{-}NH_4^+$ and for VFA contents by adding 1 mL of 25% metaphosphoric acid. Both subsamples were then stored frozen (−20 °C) until analysis.

The ruminal fluid was thawed and diluted 1:10 with distilled water, from which dilution 100 µL were used and they were deposited in test tubes, in duplicate. The determination of $N\text{-}NH_4^+$ was carried out using the protocol described by Bal, et al. [31]. The samples were incubated at room temperature for 24 h protected from light. Finally, the results were obtained by reading absorbance in a microplate spectrophotometer (PowerWave HT de BioTek, Santa Clara, CA, USA). This reading was made at 630 nanometers.

For the analysis of VFA, a gas chromatography system was used (Shimadzu Scientific Instruments AOC20s, Columbia, MD, USA) equipped with a 100-m column (column Rtx 100 m × 0.32 mm × 0.20 um column). For the chromatography conditions the oven temperature was initially set at 145 °C for 2 min, and then increased to 220 °C at 4 °C/min. The injector and the flame ionization detector were kept at 250 °C and 300 °C, respectively.

2.4. Ruminal Degradability

The rumen degradation rate of nutrients procedure described by Mehrez and Ørskov [32] was used. Briefly, the forage samples were collected on the first day of each period and dried in an oven at 60 °C. Subsequently, the samples were ground on a 3 mm sieve. From day 9 of each period, the material was introduced through the cannulas as follows: 5 g were placed in Dracon bags and incubated in the rumen for 0, 3, 6, 9, 12, 24, 48, and 72 h. Three bags were used for each incubation time: two bags of 5 cm × 10 cm and one of 10 cm × 20 cm. Bags were introduced in the rumen in reverse order so that all the bags were removed at time 0 h and then were immediately washed intensively until clear water was obtained. The bags of time 0 h were included in the last process to evaluate the loss of nutrients during the washing process.

Once washed, the bags were oven-dried at 60 °C for 48 h to calculate the DM content. Subsequently the CP, NDF and ADF were determined as previously described. The degradation rate of the nutrients (DM, CP, NDF and ADF) were determined according to the equation 1 proposed by Ørskov and McDonald [33], where p is the nutrient degraded at incubation time t, a is the rapidly degradable fraction, b is the slowly degradable fraction, and c is the degradation rate of b. The effective ruminal degradability (P) of the nutrients was evaluated using the equation 2, where k is the flow rate assumed at 6%/h [33].

$$p = a + b\,(1 - e^{-ct}) \quad (1)$$

$$P = a + bc/(c + k) \quad (2)$$

2.5. Nitrogen Use Efficiency

The collection of blood, urine and faeces followed the protocol described by Colmenero and Broderick [34]. Ten millilitres of blood were collected from each animal by puncture of the coccygeal vein, using vacuum tubes without anticoagulant with a vacutainer system. The samples were taken in the morning of the day 14 of each period, 4 h before (6:00 h) and 4 h after (12:00 h) feeding. The blood collected was centrifuged at 2.900 rpm for 15 min. The plasma obtained was stored in Eppendorf tubes and kept at −20 °C until its analysis for urea.

Urine and faeces samples were collected from the cows on the morning of day 14 of each period, 4 h before and 4 h after feeding. Ten millilitres of acidified urine were stored in 40 mL of H_2SO_4, while the faecal samples were collected in hermetic Ziplock-type bags. Both types of samples were stored at −20 °C until analysis. Plasma ureic N and urine N were determined spectrophotometrically using an automated HumaStar 200 (Human GmbH, Wiesbaden, Germany). The faecal samples were thawed at room temperature and 200 g of each were reserved and dried in an oven at 60 °C for 48 h to estimate the DM content. Then, it was passed through a 1 mm sieve and the excreted nitrogen content was estimated using the Kjeldahl method (method 2001.11; AOAC [29]).

2.6. Statistical Analysis

All statistical analyses were conducted using SAS v9.3 (SAS Institute Inc., Cary, NC, USA). To analyse the chemical and botanical composition data, an analysis of variance (ANOVA) was performed using the general linear model procedure. The morning and afternoon values of the ruminal parameters were averaged as one value per cow per period and analysed using the general linear model procedure for a Latin Square design.

Similarly, to evaluate the effect of the diets on the ruminal degradability of DM, CP, NDF and ADF along the different rumen incubation times, analysis of variance was carried out. Differences in least squares means in all linear models were investigated using the *t*-test, following Tukey's adjustment for multiple comparisons. Significant differences were assumed when $p < 0.05$ and trend when $p \leq 0.1$.

3. Results

3.1. Chemical Composition of Forage Comprising the Diets

The forage of the MIX and LEG diets had 70% lower DM content than the forage of the CON diet (Table 2). The forage of the LEG diet had the highest CP content compared to the other two diets ($p < 0.001$), and the forage of the MIX diet had higher CP content than the forage of the CON diet ($p < 0.001$). The forage of the MIX diet had 14% less NDF than the forage of the CON diet ($p < 0.001$), but 13% more than the forage of the LEG diet ($p < 0.001$). The forage of the LEG diet had 24% less NDF than the forage used in the CON diet ($p < 0.001$). The forage of the MIX diet had 14% less ADF than the forage of the CON diet ($p < 0.01$). The lignin content was 56% higher in the forage of the LEG diet than for the other two forages ($p < 0.001$). The ash content in the forage of the MIX and LEG diets were higher than that of the forage of the CON diet ($p < 0.001$).

The percentage of NDIN of the forage of the MIX diet was higher than that of the CON forage ($p < 0.01$) but lower than that of the forage of the LEG diet ($p < 0.01$). Likewise, the forage of the LEG diet presented higher NDIN than the forage of the CON diet ($p < 0.01$). The percentage of TDN of the LEG diet forage tended to have a lower TDN than the MIX and CON diets ($p = 0.09$).

The true dry matter digestibility (TDDM) of the MIX diet forage had a 5% greater TDDM than the forage from the LEG diet ($p < 0.05$), which did not differ from that of the CON diet ($p < 0.05$).

Table 2. Chemical composition of the forage fraction of the diets: CON (lucerne hay + maize silage), LEG (fresh lucerne) and MIX (fresh berseem clover/oat).

	CON	LEG	MIX	SEM	*p* Value
DM (g/kg)	546 [a]	193 [b]	217 [b]	65.7	<0.001
CP (g/kg)	102 [c]	238 [a]	149 [b]	4.2	<0.001
NDF (g/kg)	457 [a]	348 [c]	392 [b]	7.0	<0.001
ADF (g/kg)	284 [a]	274 [ab]	245 [b]	8.6	<0.01
Lignin (g/kg)	42 [b]	61 [a]	36 [b]	3.0	<0.001
Ether extract (g/kg)	23	19	18	1.5	0.10
Ash (g/kg)	65 [b]	109 [a]	105 [a]	2.8	<0.001
NDIN (g/kg)	5.0 [c]	12.1 [a]	8.8 [b]	0.98	<0.01
ADIN (g/kg)	2.9	3.8	3.8	0.77	0.36
TDN (%)	65.9	62.9	65.0	0.88	0.09
Metabolizable energy (kcal/kg)	2471	2527	2484	36.5	0.68
TDDM (%)	76.0 [a]	73.5 [b]	77.4 [a]	0.91	0.04

DM: dry matter; CP: crude protein, NDF: neutral detergent fibre; ADF: acid detergent fibre; NDIN: nitrogen insoluble in neutral detergent; ADIN: nitrogen insoluble in acid detergent; TDN: total digestible nutrient; TDDM: true digestibility of dry matter; SEM: standard error of the mean. Values in the same row with different letters differ among themselves according to the Tukey test for multiple comparisons ($p < 0.05$).

3.2. Botanical Composition of the Herbages

As described by Peede [35], the LUC pasture had a high prevalence of LUC, which remained constant during the three periods (87.5 ± 3.60 DM%; $p < 0.05$), averaging a total of 87 DM%. Even though the percentage of BC was declining over time (63.4, 44.8 and 36.6% for periods 1, 2 and 3, respectively; $p < 0.001$), this species generally dominated the BC/OAT pasture, with an average of 48 DM%. The OAT content in the BC/OAT pasture increased over time (11.0, 25.2 and 29.8% for periods 1, 2 and 3, respectively; $p < 0.01$), while the annual ryegrass and weeds contents fluctuated between periods (ryegrass: 10.6, 1.2 and 15.2% and weeds 14.8, 28.4 and 18.4% for periods 1, 2 and 3, respectively; $p < 0.01$). The ryegrass was the species that had the least content in the herbage throughout the study.

3.3. Dry Matter Intake and Ruminal Fermentation

The DMI and the ruminal pH values were similar for the three diets (Table 3). The cows feed the MIX and CON diets had similar ruminal N-NH_4^+ values, however, the cows feed the LEG diet presented a higher ($p < 0.001$) ruminal N-NH_4^+ content than the other two diets ($p < 0.001$; Table 3).

All cows presented similar total VFA, but there were slight variations in the proportions of some individual VFA. The cows feed the CON diet tended to have higher lipoacid/glucoacid and acetic/propionic acid ratios than the cows feed the LEG diet ($p = 0.10$ for both). The cows feed the CON diet tended to have a lower valeric acid proportion than the cows feed the MIX diet and a lower isobutyric acid proportion than the cows feed the LEG diet ($p = 0.06$ for both).

Table 3. Effect of diet type on the dry matter intake, volatile fatty acid (VFA) proportions, pH and ruminal ammonia in fistulated cows.

	CON	LEG	MIX	SEM	*p* Value
Dry matter intake	12.77	12.70	12.75	0.126	0.98
Ruminal pH	6.44	6.47	6.38	0.118	0.90
N-NH_4^+ (mmol/L)	6.33 [b]	14.82 [a]	7.38 [b]	0.806	<0.001
Total VFA (mmol/L)	128.5	133.1	132.6	15.86	0.96
Acetic acid (%)	71.17	68.37	69.16	0.809	0.06
Propionic acid (%)	14.18	15.58	15.13	0.560	0.13
Butyric acid (%)	9.59	10.19	10.54	0.492	0.29
Valeric acid (%)	1.31	1.58	1.63	0.127	0.05
Isobutyric acid (%)	1.27	1.59	1.37	0.102	0.07
Isovaleric acid (%)	1.88	1.98	1.75	0.157	0.32
Hexanoic acid (%)	0.67	0.67	0.64	0.060	0.82
Acetic acid/Propionic acid	5.05	4.49	4.63	0.202	0.10
Lipoacids/glucoacids ((acetic + butiric)/propionic))	5.72	5.16	5.27	0.218	0.10

CON: lucerne hay and maize silage plus concentrate; LEG: fresh lucerne plus the same concentrate as in CON; MIX: fresh berseem clover/oat plus the same concentrate as in CON. SEM: standard error of the mean. Values in the same row with different letters differ among themselves according to the Tukey test for multiple comparisons ($p < 0.05$).

3.4. *In Situ* Ruminal Degradation

The DM soluble fraction of the forage of the MIX diet did not differ from that of the LEG or CON diets, while the forage of the LEG diet tended ($p = 0.10$) to have lower DM soluble fraction than that of the CON diet (Table 4). There was no effect of the forage type on the DM slowly degradable fraction of the forages ($p > 0.05$). The forage of the LEG diet presented greater ($p < 0.05$) rate of degradation of the DM slowly degradable fraction than that of the CON diet ($p < 0.05$), while the rate of DM degradation of the slowly degradable fraction of the MIX diet forage was similar to those of the other diets ($p > 0.05$). The DM effective degradability was similar in the forage of the MIX and LEG diets, and they were higher than that of the CON diet ($p < 0.05$; Table 4, Figure 1a).

The forage of the MIX diet had 16% lower content of the soluble fraction of CP and 14% greater of the CP slowly degradable fraction than the forage of the CON diet ($p \leq 0.001$; Table 4). The CP soluble fraction of the forage of the MIX diet was 18% lower than the forage of the LEG diet ($p < 0.01$), while the slowly degradable fraction was 26% greater for the forage of the MIX diet than that of the LEG diet ($p < 0.05$). There were no differences in the degradation rates of the slowly degradable fraction of CP between the forage of the MIX and LEG diets, while the forage of the MIX diet tended to be greater than that of the CON diet ($p = 0.10$). The CP degradation rate of the slowly degradable fraction of forage of the LEG diet was greater than of the CON diet ($p < 0.05$). The effective degradability of CP was similar for the forages of the MIX and LEG diets, and they were greater than that of the CON diet ($p < 0.05$; Figure 1b).

The soluble fraction of NDF and ADF followed a similar pattern, tended to be greater in the forage of the MIX and LEG diets than that of the CON diet ($p = 0.06$ for the four comparisons), while between the forages of the MIX and LEG diets there were no differences ($p < 0.05$; Table 4). The slowly degradable fraction of NDF and ADF were similar between the forage types ($p < 0.05$). The degradation rate of the slowly degradable fraction for NDF tended to be higher in the forage of the MIX and LEG diets than that of the CON diet ($p = 0.01$ for the 2 comparisons), while there were no differences between the forages of the MIX and LEG diets ($p < 0.05$). Regarding ADF, the forage of the MIX diet did not present differences in the degradation rate with respect to the forage of the LEG and CON diets ($p < 0.05$), while the forage of the LEG diet tended to be greater than that of the CON diet ($p = 0.09$). The effective degradability of both NDF (Figure 1c) and ADF were similar between the forage of the MIX and LEG diets ($p < 0.05$), and they presented a higher effective degradability than the forage of the CON diet ($p < 0.05$).

Table 4. Ruminal degradation in situ of DM, CP, NDF and ADF of the forages used in the study.

	CON	LEG	MIX	SEM	*p* Value
DM					
a	42.4	40.8	42.1	0.47	0.11
b	46.2	46.9	50.6	2.36	0.90
c	0.033 [b]	0.116 [a]	0.080 [ab]	0.017	0.05
P	58.1 [b]	71.5 [a]	70.1 [a]	1.89	0.02
CP					
a	53.4 [a]	43.8 [b]	37.0 [c]	0.85	<0.001
b	37.5 [c]	50.4 [b]	57.2 [a]	1.20	<0.001
c	0.045 [b]	0.144 [a]	0.110 [ab]	0.016	0.03
P	69.4 [b]	78.9 [a]	73.8 [a]	1.67	0.04
NDF					
a	5.2	11.1	9.2	1.29	0.07
b	79.1	60.6	77.4	16.8	0.31
c	0.028	0.092	0.072	0.017	0.09
P	28.3 [b]	47.6 [a]	49.3 [a]	3.62	0.03
ADF					
a	0.04	3.8	3.1	1.04	0.10
b	91.8	60.6	82.2	12.00	0.36
c	0.024	0.084	0.062	0.015	0.12
P	21.6 [b]	38.9 [a]	41.6 [a]	3.12	<0.05

CON: lucerne hay and maize silage; LEG: fresh lucerne; MIX: fresh berseem clover/oat; *a*: soluble fraction, *b*: slowly degradable fraction, *c*: rate of degradation of the slowly degradable fraction, *P*: effective degradability, SEM: standard error of the mean, NS: non-significant. Values in the same row with different letters differ among themselves according to the Tukey test for multiple comparisons ($p < 0.05$).

3.5. Nitrogen Use Efficiency

The plasma ureic *N* of the cows feed the MIX and CON diets were 26% lower than that of the cows feed the LEG diet ($p < 0.05$; Table 5). The cows feed the MIX diet presented 44% less urea in urine than the cows feed the CON diet ($p < 0.05$). The percentage of faecal N did not vary between diets ($p < 0.05$).

Table 5. Effect of diet type on the plasma ureic N, urine and faecal nitrogen.

	CON	LEG	MIX	SEM	*p* Value
Plasma ureic N (mg/dL)	16.2 [b]	22.0 [a]	16.5 [b]	1.54	0.03
Urinary urea (g/d)	205 [a]	136 [ab]	114 [b]	20.3	0.04
Faecal N (%)	16.2	22.0	16.5	1.54	0.15

CON: lucerne hay and maize silage plus concentrate; LEG: fresh lucerne plus the same concentrate as in CON; MIX: fresh berseem clover/oat plus the same concentrate as in CON. SEM: standard error of the mean. Values in the same row with different letters differ among themselves, ($p < 0.05$). N: nitrogen; SEM: standard error of the mean.

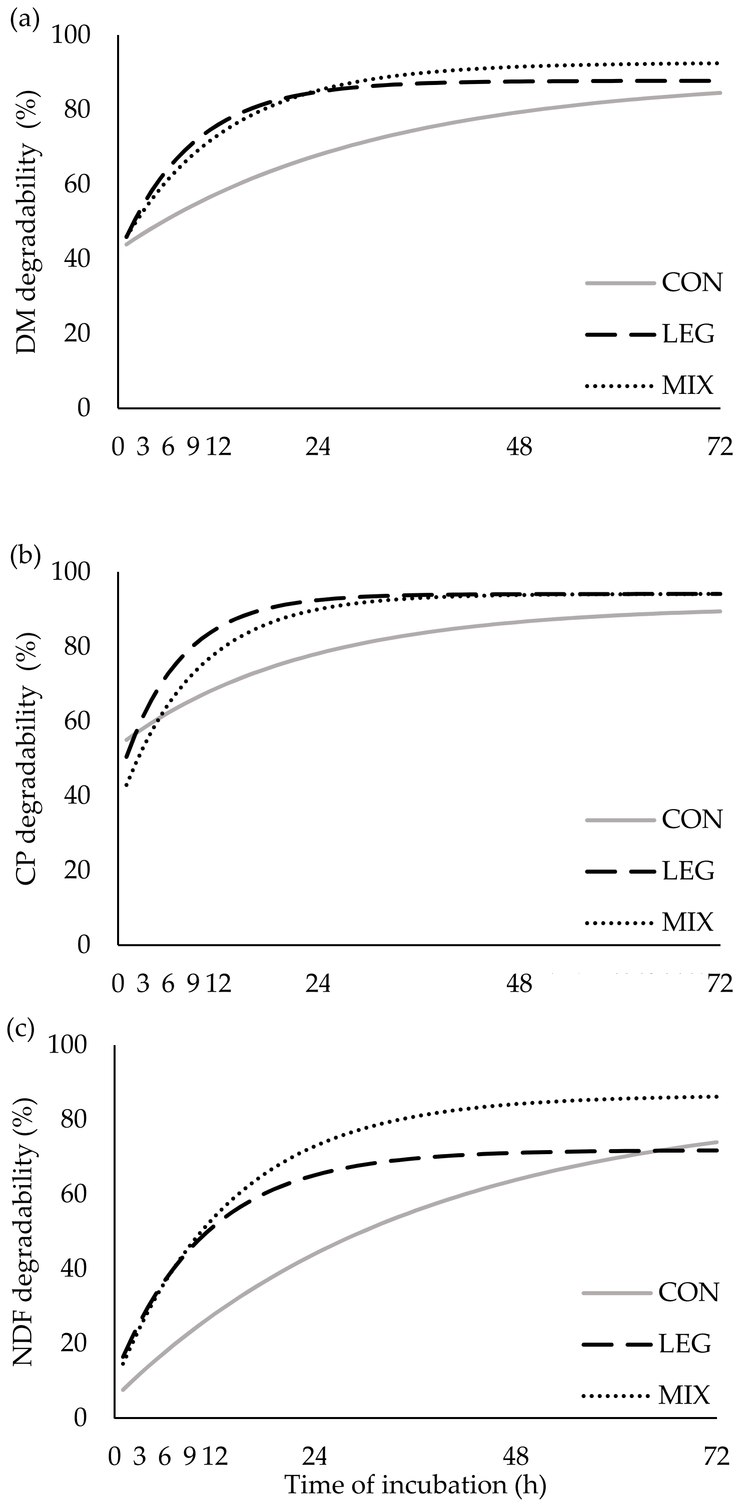

Figure 1. Degradability curves for the DM (**a**), CP (**b**) and NDF (**c**) of the forages used in the study, calculated according to the function *p* = a + bc/(c + k) of de Ørskov and McDonald [33].

4. Discussion

The overall similar rumen fermentation parameters observed among the cows feed either of three diets lead as to reject our first hypothesis. Although the forage quality of the CON treatment was lower, due to its higher concentration of NDF, compared to the treatments that included fresh forages, the ruminal pH values of the cows did not vary when they were offered the different diets. This could be due to the fact that the amount of

NDF in the diets was sufficient to promote chewing and saliva production and thus avoid the drop in ruminal pH [36], and also to the fact that NDF levels of the diets exceeded the minimum value recommended of 25% DM [28] to promote rumen health in dairy cows. On the other hand, the absence of difference in ruminal pH could be due to the similar production of total VFA between the treatments, which were within the values associated with normal levels of ruminal pH [37]. The similarity of pH and total VFA production at the ruminal level are related to the similar percentages of soluble DM observed between the different forage sources [38,39]. The observed pH values agree with that reported by Santana, et al. [40] for cows fed diets that combine legume-based forage with TMR. Similar to the values that we observed, Mendoza, et al. [14] reported neutral pH values from 6.41 to 6.53 in multiparous lactating cows fed with TMR that had between 4 to 8 h of access to fresh grass-based forage, which accounts for the benefits of the contribution of fibre from forages to stimulate salivation and thereby maintain the buffering capacity with the consequent control of ruminal pH. However, the low number of animals used in this experiment cannot be disregarded as a potential reason for the lack of differences observed for the rumen fermentation parameters.

The values of the soluble fraction of DM of the LEG and MIX treatments observed in this study are similar to those reported by Mustafa and Seguin [22] for LUC silages (41%), but greater than those reported for the BC silage (33%). In the MIX diet of our study, a mixture of BC with VA was used as a source of forage, and some studies have reported that oats in a vegetative state present 69% of soluble fraction [41], which would generate an additive effect when delivered with BC. The observed trend of a lower value of the soluble fraction of the DM of the LEG treatment could be due to its higher lignin content with respect to the forage of the CON and MIX diets, which reduced the digestibility of the forage nutrients [23]. The absence of differences in the slowly degradable fraction of the DM of the forages used in this study could be explained by the neutral values of ruminal pH found in all the treatments, which ensure the growth and activity of the rumen microorganisms and consequently, ruminal functioning and nutrient flow [42,43]. The lower speed of DM degradation of the CON treatment with respect to the LEG diet could be due to its higher content and low digestibility of NDF [23].

The effective degradability of DM of the forages of the MIX and LEG diets were similar to each other, which agrees with the study by Mustafa and Seguin [22] for BC and LUC silages (average 60%). However, in our study the ruminal degradability of the DM of the forages used in the MIX and LEG treatments was higher than that value (average 71% between the MIX and LEG treatments), which is explained by the lower fibre content of forages used fresh. In addition, the effective degradability of the forage DM was 22% higher in LEG and MIX with respect to the forage of the CON treatment. This difference probably resulted from the lower percentage of NDF in the forage of the MIX and LEG diets compared to the CON diet [44]. The latter suggest that the use of fresh forage increases the ruminal degradability of DM and other nutrients, which can lead us to accept the second hypothesis. On the other hand, even though the highest effective degradability of DM was observed for diets based on fresh forage, the three diets provided the same concentration of ME, probably due to the similar concentration of total digestible nutrients (average 65%). In general, the greater degradability of DM, NDF and ADF in the fresh forages would allow a better use of WSC from the diet [4]. However, this response was not reflected in terms of VFA concentrations.

The forage of the MIX diet presented a lower soluble fraction and a higher slowly degradable fraction of CP compared to the forage of the LEG and CON diets. Similar results were reported for BC and LUC offered as fresh forage [20] as in silage [22]. The CP degradation rate was similar for the forage of the MIX and LEG diets, which agrees with the fact that the effective degradability was similar between both forages. Contrary to our study, Mustafa and Seguin [22] reported a lower effective degradability of CP in BC silage compared to LUC silage (72% and 87%, respectively), which could be due to its higher NDIN content (5.2% and 1.8%, respectively), since insoluble protein tends to be slowly degraded in contrast with soluble protein and non-protein N [4]. The percentage of NDIN of the forages used in the LEG and MIX diets of our study (1.21 and 0.88%, respectively) were lower and less, contrasting with respect to the values reported by Mustafa and Seguin [22] for the ensiled forages, which could explain the similar CP degradation rate and CP effective degradability among the fresh forages of our study. Although the CP concentration was lower for the forage of the CON diet, this diet presented a higher soluble fraction of the CP than the forage of the MIX and LEG diets, which would be explained by the higher non-protein nitrogen content of the silages in relation to fresh forages [45]. Meanwhile, the effective degradability of CP for the forage of the MIX and LEG diets was higher than for the forage of the CON diet, which would be related to the lower rate of degradation of the slowly degradable fraction of the CP of the CON forage. Although there is a high rate of non-protein nitrogen and protein N degradation in preserved forages, the degradation rate of CP can be affected by an increase in the content of non-protein nitrogen and by a higher concentration of NDF, a situation that occurs when forages preserved are compared with fresh forages [44].

The ruminal ammonia concentration increased in the cows feed the LEG diet, reflecting this diet forage high amount of CP and the high degradability of its soluble fraction [46]. The lower concentration of ruminal ammonia observed in the cows feed the MIX diet with respect to the cows feed the LEG diet is associated with its lower CP content and lower CP soluble fraction, with a similar degradation rate between both fresh forages. These characteristics may be related to the lower proportion of protein scaping rumen degradation of LUC when compared to BC [47]. Based on the above, the expected lower concentration of ruminal ammonia in cows feed fresh forages compared to preserved forages would only be confirmed for the mix of fresh forages of BC/OAT, which would allow a better NUE with respect to animals fed with fresh LUC forage. Despite the higher ruminal ammonia content observed with the LEG diet, this was not reflected in the content of short-chain VFA, precursors of its synthesis. The fact that the fresh forage diets supplied more CP added to their greater effective degradability would probably explain this effect [46,48]. The higher rumen ammonia content and a trend towards higher isobutyric acid observed in the cows feed the LEG diet suggest insufficient synchronization in the energy inputs and CP available for the synthesis of microbial protein, associated with a higher content of degradable protein in the rumen and a low energy intake [49]. The higher level of ruminal ammonia in the cows feed the LEG diet is consistent with the levels of plasma ureic N and the greater CP content and its higher CP effective degradability that was observed for the forages of the LEG diet when compared with those of the other diets. However, the cows that presented the highest concentration of urea in the urine were those feed with the CON diet, which is inconsistent with what was previously described, so it is not clear why this effect occurred. Meanwhile, contrary to what was found by Enriquez-Hidalgo, et al. [8] when comparing similar diets as CON and MIX, the NUE related results presented here suggest that the MIX diet can improve cows' NUE, leading us to only partially accept our third hypothesis.

No differences were found in the soluble, slowly degradable fractions or in the degradation rate of NDF between the diets' forages, which could also be explained by the similar DM intake of treatments [46]. Mustafa and Seguin [22] reported a lower soluble fraction and a higher slowly degradable fraction of NDF for BC silage compared to LUC silage, despite the fact that there were no differences in the degradation rate between both forages. However, BC was used as pure forage preserved as silage and not mixed with OAT offered fresh to the animals as occurred in our study. The high content of cellulose and fermentable hemicellulose of both BC [50] and OAT [51], added to the greater degradability that OAT presents when compared to LUC [41], could explain the similar ruminal kinetic parameters of NDF observed between the MIX and LEG diets' forages.

The highest level of fibre degradability occurred when the cows were fed fresh forages, with a higher contribution of CP compared to the CON treatment, which favours the growth and activity of cellulolytic bacteria [40,52] and improves fibre digestibility. Similarly, branched chain VFA (a mixture of isobutyric, 2-methylbutyric and isovaleric acids) from feed protein degradation in the rumen are used for the development of these bacteria [53]. The slightly higher percentage of acetic acid in the CON diet, together with a slightly higher ratio of acetic: propionic acids and lipoacids/glucoacids with respect to the LEG diet, could be explained by the higher content of NDF found in the preserved forage of the CON diet, which could potentially induce a higher milk fat content in lactating dairy cows [54].

5. Conclusions

The use of fresh forages of lucerne or berseem clover mixed with oats as a source of forage for the TMR of cows had almost no effects on ruminal characteristics such as pH and VFA, but the lucerne forage increased the concentration of ammonia and both improved the ruminal kinetics of DM and CP. Degradability of DM, CP, NDF and ADF increased both with the use of LUC and with the BC plus OAT.

The MIX diet decreased serum N urea compared to the LEG diet and the urinary urea concentration compared to the CON diet. The results suggest that the use of BC with OAT in a fresh form offers a good option in terms of NUE, since it presents a lower soluble CP and greater ruminal degradability. Thus, it would be expected to find more protein available for animal metabolism, which would not be expected with the use of fresh LUC.

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Data Availability Statement: The datasets generated for this study are available on request to the corresponding authors.

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