

DETERMINATION OF NUTRIENT DIGESTIBILITY OF TOTAL MIXED RATIONS IN THE RUMEN USING *IN VITRO* METHOD*

Dženan Hadžić^{*1}, Hana Husejnagić², Senada Čengić-Džomba¹, Emir Džomba¹

Original scientific paper

Abstract

The digestibility rate is a crucial indicator of nutrient utilization in dairy cow diets. We can accurately determine ruminant feed digestibility in an artificial rumen using *in vitro* fermentation. This involves incubating a feed sample with liquid rumen under anaerobic conditions using a buffer solution that simulates rumen saliva. After approximately 48 hours of fermentation, the remaining dry matter was measured, followed by the quantification of digested cellulose, produced gases, and volatile fatty acids. Commercial complete rations (TMR) were used in the experiment to ensure uniform consumption in both quantitative and qualitative terms. The research aimed to determine the digestibility rate and kinetics of dry matter and nutrient components in TMR samples designed for milk production in different lactation stages. The results showed significant differences in the composition of the mixtures and the same indicators after incubation through eight different time intervals. The research confirmed that digestibility analysis with the ANKOM incubator provided accurate, reproducible, and cost-effective results. Collaborative studies also yielded desirable results despite differences in fermentation factors.

Keywords: *In vitro* digestibility, total mixed rations (TMR), dairy cows

INTRODUCTION

Ruminants, such as cattle, can efficiently digest rations rich in coarse forage due to their unique digestive system. Ruminal fermentation is a continuous process due to the constant turnover of microorganisms in the rumen (Valentine et al., 1994). The *in vitro* dry matter digestibility (IVDMD) method is commonly used to evaluate the nutritional value of ruminant feeds. As a closed system, the *in vitro* digestibility method uses simulated digestive fluids and enzymes to study feed digestion. An artificial incubator with a thermostatic chamber at 39°C and four rotating jars was used to measure the *in vitro* digestibility of feeds. The Ankom Daisy[®] (Ankom Technology, Macedon, NY, USA) apparatus allows simultaneous incubation of different feedstuffs in sealed bags, making the process more efficient. The method is based on Tilly and Terry's (1963) and

* Paper presented at the 34th International Scientific-Expert Conference of Agriculture and Food Industry, October 9-11, 2024, Sarajevo, Bosnia and Herzegovina

¹ University of Sarajevo, Faculty of Agriculture and Food Sciences, Sarajevo, B&H

² MA student of Faculty of Agriculture and Food Sciences, Sarajevo, B&H

IVDMD. Since then, the technique has been modified (Goering and Van Soest, 1970; Ammar *et al.*, 1999). Modifications utilized by various researchers include different rumen (fluid) inoculum, buffer solutions, sample size and weight, and bag type, which have been developed to adjust the pH of the inoculum and equilibrate fermentation processes (Mabjeesh *et al.*, 2000). Due to costs, labor-intensive and ethical considerations, alternative *in vitro* analysis is preferred and widely used (Tassone *et al.*, 2020). It provides accurate data for predicting ruminant digestibility. This scientific work focuses on researching the digestibility rates and kinetics of dry matter and individual nutrient components in samples of commercial TMR rations in the rumen using the *in vitro* method.

MATERIALS AND METHODS

The study utilized original commercial total mixed ration (TMR) samples and rumen fluid, collected from lactating cows at various stages, obtained from the dairy farm “KJP PD Bojnik” in Sarajevo (<https://pdbutmir.com>). These rations were formulated to meet their maintenance and production requirements (NRC, 2001). The TMR rations were composed of corn silage, grass-legume mixture hay, alfalfa haylage, concentrate mixture (22% crude protein), super concentrate mixture (38% CP), brewers grains, limestone, salt, sodium bicarbonate and premix (Table 1). All preparations and procedures were conducted at the Animal Feeds and Feeding laboratory of the Faculty of Agriculture and Food Sciences (University of Sarajevo). The pre-dried samples (at 55°C for 16 hours) were finely ground in a Foss Hammertec™ hammer (Foss Analytics, Hillerød, Denmark) mill through a ~2.0 mm screen. Approximately 0.5±0.05 g of the sample was weighed, placed, and encapsulated (heat sealed) in the filter (acetone pre-rinsed) bags (F57, polyester, with a porosity of 25 µm). Three Simmental dairy cows were used as rumen fluid donors (with one cow representing each of the three production phases). Rumen fluid was collected using a vacuum pump connected to a glass container and fitted with a semiflexible ~1.8-meter-long oro-ruminal probe. The probe was inserted into the oro-ruminal tract within the ventral sac of the rumen. Approximately 2,0 liters of rumen inoculum was collected using the UPS-NS vacuum esophageal probe (Lalić *et al.*, 2001) using the probing technique 4 hours after the morning consumption. The initial 500 ml of inoculum was discarded because of potential saliva contamination (Raun and Burroughs, 1962). The pH of the rumen liquor was measured right after collection, and it was found to be 6.67. The liquid content of the rumen was transferred to a sterile, prewarmed (40.0±1.0 °C) container and immediately transported to the laboratory. Up to 25 samples were transferred into each digestion jar. Then, 400 ml of inoculum (pre-filtered through four layers of cheesecloth) was added to the buffer solution (artificial saliva). Buffer solutions A (KH₂PO₄ (10.0 g/L), MgSO₄×7H₂O (0.5 g/L), NaCl (0.5 g/L), CaCl₂×2H₂O (0.1 g/L), Urea (0.5 g/L)) and B (Na₂CO₃ (15.0 g/L), Na₂S×9H₂O (1.0 g/L)) are prepared by combining 266 ml of solution B with 1330 ml of solution A in a 1:5 ratio to create a total volume of 1600 ml. The mixture was purged with CO₂ gas for thirty seconds and the lid (with a check valve) was sealed. The

prepared jars were incubated at $39 \pm 0,5$ °C for 3, 6, 12, 24, 48, 72, and 96 hours (Daisy II Incubator, Ankom Technology, Macedon, NY, USA). Each sample was incubated in three replicates. After incubation, the bags are removed and rinsed in tap (cold) water.

Also, applying a Neutral Detergent Solution is essential to remove any microbial debris or remaining soluble fractions. Then, they are soaked in acetone, rinsed again and dried in an oven at 105°C until a constant mass is achieved (up to two hours). The bags are then weighed and the *in vitro* dry matter digestibility (IVDMD) is calculated based on the amount of dry matter that disappeared from the initial weight inserted into the bag.

Samples of complete (TMR) mixtures were analyzed before and after incubation to determine the content of individual nutrients. The analyses included determining dry matter (DM) and moisture content (TM) by drying (Memmert UE 500; ISO 6496:1999), crude ash (Ash) and organic matter (OM) by incineration (Nabertherm 7/H; 5984:2002), crude protein (CP) by combustion (Foss TecatorTM; ISO 5983-1:2005), distillation and titration (KjeltecTM 2200; 5983-2:2005) and neutral (sodium sulfite; α -amylase) and acid detergent fiber (ADF) content by extraction (Ancom A200; ISO 16472:2006 and 13906:2008) based on Van Soest et al. (1991) and Mertens (2002).

Starch (ST) content was determined polarimetrically (ISO 6493:2000). Calculation of *in vitro* DM, CP, NDF and ADF digestibility content was performed using the following formula (1), where W_1 is bag mass (g), W_2 is sample mass (g), W_3 is bag and sample residual mass (g), C_1 is blank bag correction (final/initial bag mass).

$$(1) \quad IVDMD = \frac{100 - (W_3 - (W_1 \times C_1)) \times 100}{W_2}$$

Table 1. Ingredients, chemical composition and daily intake of commercial TMR rations fed to the cows on a farm

Ingredient composition (% DM⁵)	1st phase of lactation	2nd phase of lactation	3rd phase of lactation
Corn silage	40.6	45.5	54.9
Grass-legume mix. hay	11.7	17.2	19.2
Alfalfa haylage	2.2	6.9	10.8
Concentrate mixture (22% CP) ¹	34.8	29.1	13.6
Concentrate mixture (38% CP) ²	3.9	-	-
Brewers grains	5.5	-	-
Limestone	0.1	0.3	0.4
Salt	0.3	0.2	0.2
Sodium bicarbonate	0.2	-	-
Premix ³	0.7	0.8	0.9
F:C ⁴	54:46	69:31	85:15
Chemical composition (% of DM)			
Dry matter	47.2	41.8	40.5
Crude ash	6.61	6.33	5.83
Organic matter	93.39	93.67	94.17
Crude protein	12.93	10.17	9.08
Neutral detergent fiber	52.26	50.88	53.39
Acid detergent fiber	21.97	22.10	21.58
Starch	30.06	34.77	30.14
Daily intake (g/day)			
Dry matter	22.700	18.200	16.300
Crude protein	2.950	1.850	1.480
Neutral detergent fiber	11.863	9.260	8.705
Acid detergent fiber	4.987	4.022	3.517
Starch	6.825	6.328	4.913

¹cereal, by-products of the oil and fermentation industry, by-products of the mill industry, dried plant products, macroelements, microelements, vitamins; crude protein min. 22%, crude fiber max. 12%, ash max. 10%, Ca 0,72%, P 0,92%, Na 0,20%, Vit.A 10.000 IU/kg, Vit.D₃ 2.000 IU/kg, Vit. E 20 mg/kg;

²by-products of the mill industry, oilseed meal, alfalfa meal, salt, limestone, dicalcium phosphate, mineral and vitamin supplement, crude protein min. 37%, crude fiber max. 10%, ash max. 12%, P min. 1,8%, Na min. 0,6%, vit.A 30.000 IU, vit.D₃ 4.000 IU, vit.E 50 mg, Fe 80 mg, Cu 15 mg, Mn 50 mg, Zn 50 mg, Mg 80 mg, I 1,8 mg, Se 0,3 mg, Co 0,2 mg;

³Vitamins, methionine, salt, monocalcium phosphate, magnesium oxide, limestone, sugar, magnesium sulfate, Na 5,0%, P 1,0%, Ca min. 15%, Mg 3%, S 0,5%, methionin 1%, sugar 5%, vit.A 220.000 IU, vit.D₃ 40.000 IU, vit.E 1.500 mg, vit.B₁ 21 mg, vit.B₂ 10 mg, vit.B₆ 4 mg, vit.B₁₂ 0.5 mg;

⁴forage to concentrate ratio;

⁵DM – dry matter

The pH of the ruminal fluid was measured immediately after each incubation endpoint (pH700 Eutech Instruments, Singapore). Table 1 shows TMR's ingredients, structure, chemical composition, and estimated consumption of complete rations (based on dry matter). The obtained data were analyzed using descriptive and comparative methods using a statistical software package (IBM SPSS Statistics *ver.* 23.0). The feed and animal nutrition laboratory conducted complete sample preparation and analyses.

RESULTS AND DISCUSSION

The entire process of setting up the experimental trial was carried out according to the guidelines provided by the manufacturer of the Daisy incubator. This involved optimizing various environmental factors such as temperature, anaerobic conditions, mixing, and the amount and ratio of the artificial saliva-buffer solution to ensure proper and continuous fermentation. This is supported by the fact that the pH value did not deviate significantly from the usual values. The initial value (6.60) did not change compared to the endpoint of incubation (96 hours), ranging from 6.38 to 6.60 throughout the studies shown in Table 2. The digestibility of dry matter is a critical factor in determining the nutritional value of a feed. The results for the IVDMD revealed significant variations in complete meals during incubation (Table 2). The values ranged from 25.84 to 27.20% at the third hour and increased to 77.48% and 79.58% by the 96th hour of measurement. Tassone *et al.* (2023) published similar results in their 48-hour dry matter digestibility research. No significant differences were observed among the meals during specific measurement periods. Kahraman *et al.* (2023) reported a slight decrease in total mixed rations (TMR) digestibility when different feed ingredients were used to prepare mixed rations. Furthermore, Paul *et al.* (2023) observed a more significant decrease in the digestibility of TMR during the 24-hour and 48-hour incubation periods compared to the findings of this study.

Table 2. Average values of in vitro digestibility of DM, CP, NDF and ADF throughout the incubation period (mean±sd)

Item	Period of incubation							P*
Hour	3 ⁰⁰	6 ⁰⁰	12 ⁰⁰	24 ⁰⁰	48 ⁰⁰	72 ⁰⁰	96 ⁰⁰	
pH	6.48	6.50	6.49	6.48	6.47	6.38	6.60	
Digestibility coefficient of dry matter (%)								
TMR ₁ (n=6)	27.20 ^{aA} ±0.87	34.59 ^{bA} ±1.31	53.45 ^{cA} ±1.46	57.35 ^{dA} ±0.77	66.84 ^{eA} ±1.34	70.74 ^{fA} ±0.21	77.48 ^{gA} ±1.02	0.000
TMR ₂ (n=6)	27.10 ^{aA} ±0.77	30.60 ^{bA} ±4.34	50.43 ^{cA} ±3.03	57.49 ^{dA} ±0.44	68.04 ^{eA} ±2.16	71.27 ^{fA} ±0.52	78.73 ^{gA} ±1.22	0.000
TMR ₃ (n=6)	25.84 ^{aA} ±1.40	33.16 ^{bA} ±0.62	51.17 ^{cA} ±1.22	54.79 ^{dA} ±2.95	66.59 ^{eA} ±0.51	70.80 ^{fA} ±0.76	79.58 ^{gA} ±1.34	0.000
P**	0.282	0.252	0.254	0.192	0.489	0.477	0.181	

Digestibility coefficient of crude protein (%)								
TMR₁ (n=6)	33.63 ^{aA} ±1.44	34.28 ^{aA} ±0.95	38.19 ^{bA} ±0.57	49.14 ^{bA} ±1.89	53.98 ^{cA} ±1.59	66.57 ^{dA} ±1.32	85.74 ^{cA} ±1.46	0.000
TMR₂ (n=6)	32.09 ^{aA} ±1.57	32.33 ^{aA} ±0.74	35.90 ^{bB} ±0.88	38.66 ^{cA} ±1.30	51.80 ^{dA} ±1.72	66.96 ^{fA} ±1.04	84.39 ^{gA} ±1.35	0.000
TMR₃ (n=6)	15.76 ^{aB} ±0.70	16.02 ^{aB} ±1.43	18.17 ^{aC} ±0.59	29.33 ^{bB} ±0.93	46.22 ^{cB} ±0.97	62.94 ^{dA} ±2.91	84.61 ^{fA} ±1.36	0.000
P^{**}	0.000	0.089	0.000	0.000	0.002	0.082	0.492	
Digestibility coefficient of neutral detergent fiber (%)								
TMR₁ (n=6)	8.35 ^{aA} ±1.44	14.40 ^{bA} ±1.84	32.86 ^{cA} ±2.40	43.07 ^{dA} ±0.48	55.68 ^{eA} ±0.80	57.71 ^{eA} ±0.27	63.31 ^{fA} ±2.55	0.000
TMR₂ (n=6)	3.67 ^{aB} ±1.23	11.48 ^{bAB} ±1.63	27.39 ^{cB} ±1.32	36.05 ^{dB} ±0.71	55.04 ^{eA} ±3.88	57.70 ^{eA} ±2.48	65.87 ^{fA} ±1.96	0.000
TMR₃ (n=6)	3.89 ^{aB} ±1.74	10.91 ^{bB} ±1.55	29.83 ^{cAB} ±0.93	35.06 ^{dB} ±0.91	53.39 ^{eA} ±1.78	60.42 ^{fA} ±0.98	66.50 ^{gA} ±2.49	0.000
P^{**}	0.011	0.089	0.020	0.000	0.548	0.121	0.538	
Digestibility coefficient of acid detergent fiber (%)								
TMR₁ (n=6)	-	-	15.56 ^{aA} ±1.60	20.98 ^{bA} ±0.44	33.86 ^{cA} ±1.76	38.22 ^{dA} ±1.65	45.89 ^{cA} ±0.73	0.000
TMR₂ (n=6)	-	-	12.82 ^{aB} ±1.09	17.03 ^{bB} ±0.46	34.86 ^{cA} ±3.00	39.70 ^{dA} ±4.64	51.38 ^{fB} ±1.54	0.000
TMR₃ (n=6)	-	-	6.85 ^{aC} ±0.29	16.59 ^{bB} ±1.47	34.63 ^{cA} ±3.44	40.93 ^{dA} ±1.01	54.22 ^{fC} ±1.57	0.000
P^{**}	-	-	0.000	0.002	0.904	0.553	0.001	

DM – dry matter; CP – crude protein; NDF – neutral detergent fiber; ADF – acid detergent fiber; TMR_{1,2,3} – total mixed rations according to the phase of lactation (early, mid and late)

* The mean difference between incubation periods (Significance was declared at $P < 0.05$)

** The mean difference between TMR rations inside incubation periods (Significance was declared at $P < 0.05$)

Figure 1 illustrates the dynamics of dry matter digestibility and associated regression models, which exhibit a very high coefficient of determination (R^2). No differences between dry matter digestibility (DMD) and TMR rations were observed for any time point (Table 2).

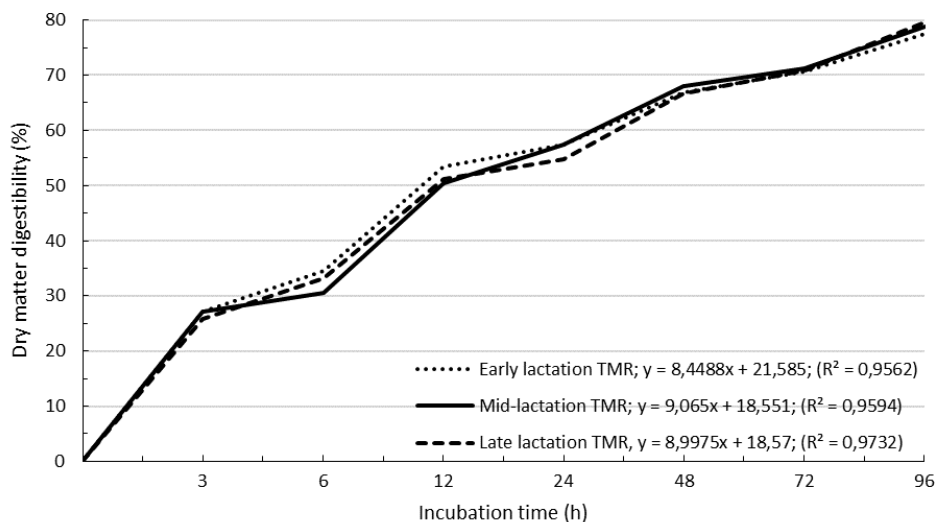


Figure 1. Dry matter digestibility rates of different TMR rations

The crude protein content in feed mixtures is primarily determined by the nitrogen levels needed to meet the requirements of dairy cows for synthesizing body and milk protein. By incubating samples, we assessed the average raw protein digestibility in the tested TMR throughout the incubation period. The results showed significant variations in crude protein digestibility among the complete meals during the incubation (Table 2). The digestibility values ranged from 32.69%, 30.34%, and 15.84% at the third hour to 85.89%, 83.58%, and 82.98% at the 96th hour of incubation for TMR₁, TMR₂, and TMR₃, respectively. The first and second TMR mixtures consistently exhibited higher raw protein digestibility throughout the incubation period. This can be attributed to the differences in soluble protein content in the mixtures before incubation and the substantial contribution of protein from concentrates, which is naturally more digestible. In contrast, the crude protein digestibility of the third TMR mixture is hindered by a significant amount of crude protein being bound to structural carbohydrates due to the more substantial proportion of bulky feed components, making it less accessible. Although this study did not cover this, it would be valuable to investigate the individual crude protein fractions and their digestibility. Furthermore, the digestibility of raw protein in the rumen is influenced by the level of productivity and intensity, which are closely tied to the retention time of feed in the rumen and the quality and physical characteristics of feed particles (including size and distribution of effective fibers). An evident difference existed in all rations between the defined incubation periods ($P < 0.05$). Significant differences in the digestibility of crude protein between meals were determined, except in the 72nd and 96th hour of incubation ($P > 0.05$). In most incubation points, differences did not exist between the first two rations, while differences appeared mainly between the first, second and third rations. The following

figure 2 illustrates the dynamics of raw protein digestibility and includes associated regression models with a high coefficient of determination (R^2).

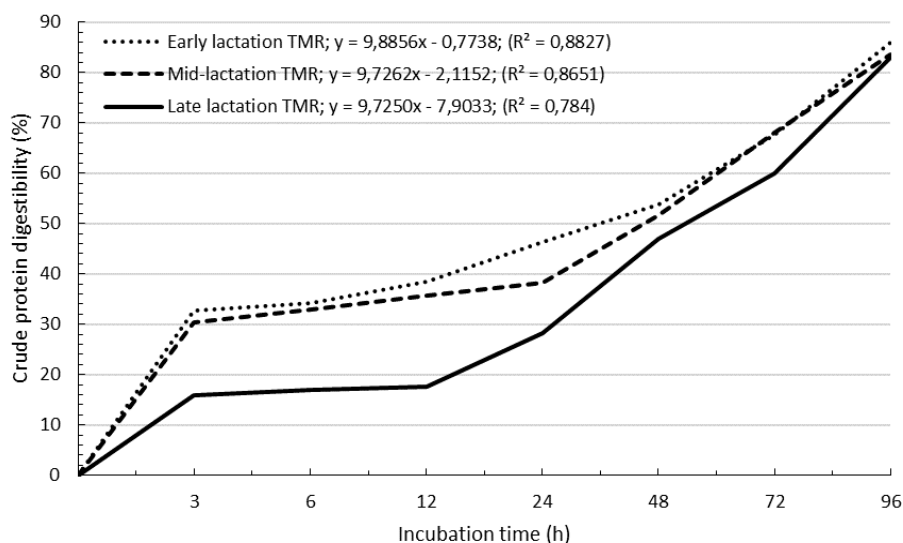


Figure 2. Crude protein digestibility rates of different TMR rations

The stagnation in the digestibility of raw protein from the 3rd to the 12th hour of incubation is closely associated with the digestibility of NDF and ADF, which refers to the protein bound to fibers (B fraction). A significant positive trend in digestibility continues to increase until the 96th hour, reaching approximately 80% across all three meals (Figures 2, 3, and 4).

Neutral detergent fibers (NDF) are the most common measure and represent the more easily digestible fraction of structural fibers, specifically hemicellulose (Mertens, 1977). The nutritional value of a ruminant feed or mixture is assessed based on its NDF content and potential digestibility. These factors significantly depend on the developmental stage of the plant material and the preservation method used. Previous studies on total mixed ration (TMR) consumption in cows indicate that mixtures with higher NDF digestibility lead to greater dry matter intake, positively correlated with higher milk yields (Oba and Allen, 1999; Spanghero and Zanfi, 2009). Notably, the results from the NDF digestibility tests showed significant variations among complete meals throughout the incubation period (Table 2). The values ranged from 8.52%, 3.75%, and 4.29% during the third hour to 63.88%, 65.88%, and 67.63% in the 96th hour of incubation. High-producing cows usually retain feed particles in their rumen for no more than 48 hours. However, recent measurements suggest that this duration may have decreased to 24 and 30 hours. This variation depends on the intensity of the cow's production and the

quality (digestibility) of the forages they consume (Oba and Allen 1999). It is explained that shorter incubation times are more effective in assessing the digestion potential of NDF in high-producing lactating dairy cows. Compared to reference values for TMR rations, these results categorize the rations as medium digestible (54.4% - 57.1%), according to Hoffman and Combs (2008). As shown in Table 2, the NDF digestibility is significantly lower in TMRs consumed by cows in the third stage of lactation due to a higher proportion of the fibrous component in the diet. Therefore, these rations are classified as poorly digestible based on reference values (45.7% - 53.9%). However, by the 72nd and 96th hours of incubation, the digestibility significantly improves for the third TMR ration. This increase may be attributed to the longer retention time of the meals in the rumen, which enhances the digestibility of both dry matter and NDF due to prolonged exposure to decomposition processes (microbial activity). The following figure illustrates the dynamics of NDF digestibility, featuring associated regression models with a very high coefficient of determination (R^2).

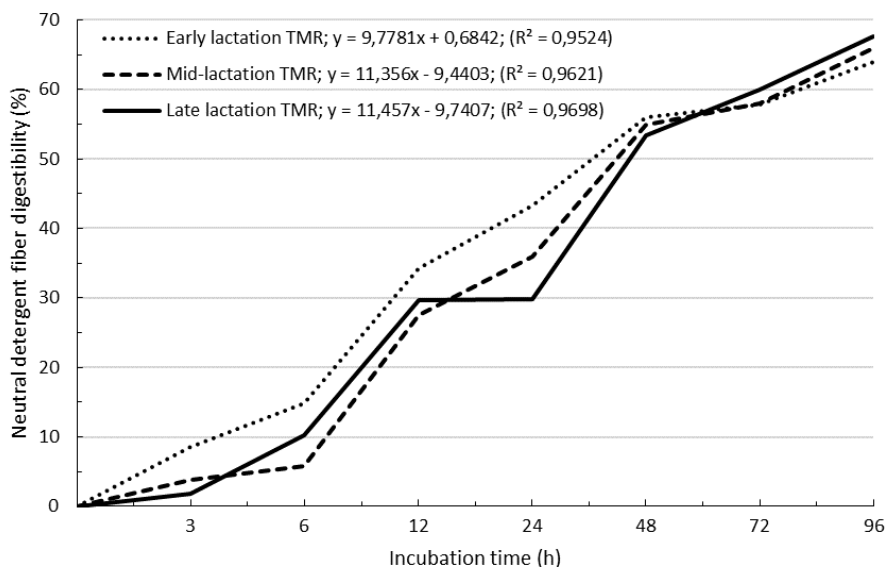


Figure 3. NDF digestibility rates of different TMR rations

The recommendations for ADF are primarily based on the needs of NDF due to their close relationship. Given the strong correlation, ADF and NDF, along with energy values, can be calculated using regression equations. Measuring ADF is essential when assessing forages of meager nutritional value, such as poor-quality hay or straw. Acid detergent fibers (ADF) represent the heavier and insoluble fiber fraction, primarily cellulose and lignin. Consequently, ADF is considered a reliable indicator of the potential digestibility of feeds or their mixtures. As anticipated, the results of ADF

digestibility revealed significant variations among the complete meals throughout the incubation period (Table 2). The digestibility values ranged from 15.37%, 12.81%, and 6.70% at the 12th hour to 45.56%, 51.48%, and 54.22% at the 96th hour. It is important to note that digestibility values for the third and sixth hours of incubation are missing. ADF tends to show measurable values only in the later stages of incubation, beginning at the 12th hour. Furthermore, there is a clear positive trend in digestibility, demonstrating significant differences between the rations across various incubation periods (figure 4).

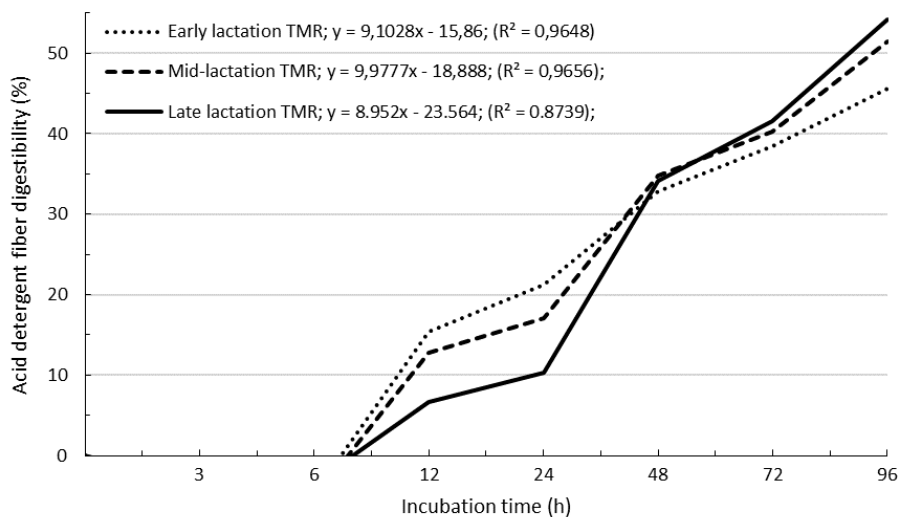


Figure 4. ADF digestibility rates of different TMR rations

CONCLUSIONS

The *in vitro* digestibility method was developed as a cost-effective and ethical alternative to the more expensive and time-consuming *in vivo* method for evaluating nutrient digestibility in ruminants and non-ruminants. This study confirms significant correlations established in some earlier research. Regarding the quality of total mixed rations, particularly for cows in peak lactation, it was noted that their crude protein content is slightly lower than the recommended for optimal production (NRC, 2001). As anticipated, the results demonstrated notable differences in the chemical composition of the complete mixtures. Additionally, variations were observed in critical indicators following incubation over eight different time intervals, affecting dry matter, crude protein and neutral and acidic detergent fibers. Based on the research conducted on digestibility and nutritional value assessment, it is evident that there is potential for

enhancing the quality of feed, particularly for those used during the first and second stages of lactation.

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ODREĐIVANJE PROBAVLJIVOSTI HRANJIVIH MATERIJAKOMPLETNIH OBROKA U BURAGU *IN VITRO* METODOM

Sažetak

Stepen probavljivost predstavlja najbitniji pokazatelj iskorištenosti hranjivih materija u ishrani mliječnih krava. U vještačkom buragu, upotrebom *in vitro* fermentacije, moguće je relativno precizno odrediti probavljivost krmiva za preživare. Ovaj postupak uključuje inkubaciju uzorka krmiva s tekućim sadržajem buraga u anaerobnim uslovima. Kao medij koristi se puforni rastvor koji simulira sastav pljuvačke u buragu. Fermentacija traje otprilike 48 sati, nakon čega se mjeri preostala suha materija, probavljena celuloza, proizvedeni plinovi i hlapljive masne kiseline. Kao materijal u radu, korišteni su komercijalni kompletni obroci (TMR) za ishranu krava, što omogućuje da konzumacija bude ujednačenja u kvantitativnom i kvalitativnom smislu. Ideja istraživanja je bila da se utvrdi stepen i kinetika probavljivosti suhe materije i pojedinačnih hranjivih komponenata u uzorcima komercijalnih TMR (kompletno miješanih) obroka, dizajniranih prema realnim proizvodnim potrebama proizvodnje mlijeka u prvom, drugom i trećem stadiju laktacije. Rezultati su pokazali značajne razlike u fizičko-hemijskim sastavu kompletnih smjesa, ali i u istim pokazateljima nakon inkubacije kroz osam različitih vremenskih intervala kako za suhu materiju, tako i za sirovi protein, neutralna i kisela deterdžentska vlakna. Ovo istraživanje je samo potvrdilo da analiza probavljivosti sa ANKOM inkubatorom je pokazala zavidne

rezultate po pitanju tačnosti, ponovljivosti i troškova sprovođenja generalno. Mnoge kolaborativne i simultane studije pokazale su zavidne rezultate, uprkos činjenici što postoje značajne razlike u pogledu osiguranja biotskih i abiotskih faktora fermentacije.

Ključne riječi: *probavljivost in vitro, ukupni miješani obroci (TMR), mliječne krave*