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Study on the measurement of nutrients utilization efficiency in Ruminants

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Study on the measurement of nutrients utilization efficiency in Ruminants

Marker-based approach to estimate apparent and true total tract nutrient digestibility in ruminants: comparative analysis and practical implications for nitrogen balance

"Your science is as good as your markers are..."

Prof. Mike Van Amburgh

Abstract

This study evaluates the effectiveness of internal markers, specifically uNDF, uNDFom, and AIA, in estimating total-tract apparent digestibility (ttaD) and total-tract digestibility (ttD) across various ruminant species and conditions. Five trials were conducted focusing on small ruminants, dairy buffaloes, dairy cows, and beef cattle, with a final trial providing a comprehensive discussion on the technical application of uNDF as marker to assess Nitrogen (N) balance (NB), and marker utilization in dairy cows fed hay-based diets within the Parmigiano Reggiano region.

In Trial 1, total collection, uNDF, uNDFom, and AIA were compared in small ruminants. The results demonstrated significant differences among markers, with uNDF providing more consistent and reliable estimates, while AIA tended to predict lower values of digestibility, compared to total collection.

In Trial 2, digestibility in Mediterranean buffaloes was analyzed. The uNDF and AIA were used as markers, with AIA producing higher estimates. However, due to variability in ash content across diets and in accordance with literature and the following study, uNDF was considered a potential option for providing more accurate fiber digestibility predictions.

In Trial 3, the comparison of uNDF and uNDFom markers was extended to dairy cows. Both markers performed similarly across most nutrients, but uNDFom offered a slight advantage in accuracy when estimating fiber digestibility, making it the preferred marker for high-fiber diet.

Trial 4 focused on beef cattle, comparing tta of dry matter (ttaDMD) when estimated by uNDF, ADL, and ADIA. The findings suggest that uNDF could again provide more accurate estimates in fiber-rich diets between the three markers, being the experimentally less variable and again aligning with literature.

Trial 5 provided a conclusive assessment of NB in dairy cows fed hay-based diets, specifically within the Parmigiano Reggiano production system, starting from markers-based calculation of

total fecal and urine output. The study highlighted the challenges of using hay-based diets, which resulted in a negative nitrogen balance (-60.77 g/day), reflecting inefficiencies in N utilization, but also focusing on the potential technical bias that can be due to inappropriate choice, or use, of markers to estimate total fecal and urine output, namely uNDF and creatinine.

In the technical annexes I and II the potential impact of urine acidification on creatinine measurement, used as marker for total urinary N output estimation, and the evaluation of an *in vitro* method to assess the digestibility of rumen-protected amino acids are briefly described and discussed. In the first annex, it is suggested that, while acidification may alter creatinine concentration, the method could remain valid for estimating urine output.

In the second annex, an *in vitro* method for ruminal and intestinal digestibility was used to evaluate a novel chitosan-based biopolymer as an alternative encapsulating agent for methionine.

In conclusion, uNDF and uNDFom are reliable markers for estimating nutrient digestibility, particularly in fiber-rich diets, while AIA may introduce variability due to ash content; the choice of marker needs to reflect the species of interest, the dietary preferences, the diet composition, and the sampling protocol. These findings are critical for optimizing feeding strategies and improving N utilization efficiency in ruminants, especially in systems heavily reliant on hay-based diets.

Keywords: nutrient digestibility, faecal marker, ruminant nutrition, nitrogen balance.

Index

List of abbreviations

AIA: Acid Insoluble Ash

ADIA: Acid Detergent Insoluble Ash

ADICP: Acid Detergent Insoluble Crude Protein

ADL: Acid Detergent Lignin

ADF: Acid Detergent Fiber

ADFom: Acid Detergent Fiber (expressed exclusive of residual ash)

aNDF: Neutral Detergent Fiber (assayed with heat-stable amylase without sodium sulfite and expressed inclusive of residual ash)

aNDFom: Neutral Detergent Fiber (assayed with heat-stable amylase without sodium sulfite and expressed exclusive of residual ash)

BOD: Biological Oxygen Demand

CH: Chitosan-based biopolymer

CP: Crude Protein

DM: Dry Matter

FTIR: Fourier-Transform Infrared Spectroscopy

Met: Methionine

NDICP: Neutral Detergent Insoluble Crude Protein

NDF: Neutral Detergent Fiber

NIR: Near Infrared Reflectance Spectroscopy

SolCP: Soluble Crude Protein

ThOD: Theoretical Oxygen Demand

tt: Total-Tract

tta: Total-Tract Apparent

ttaCPD: Total-Tract Apparent Digestibility of Crude Protein

ttaDMD: Total-Tract Apparent Digestibility of Dry Matter

ttaSolCPD: Total-Tract Apparent Digestibility of Soluble Crude Protein

ttNDFD: Total-Tract Digestibility of Neutral Detergent Fiber

ttADFD: Total-Tract Digestibility of Acid Detergent Fiber

ttCelD: Total-Tract Digestibility of Cellulose

ttHemicelD: Total-Tract Digestibility of Hemicellulose

USG: Urine Specific Gravity

VP: 2-vinyl pyridine/styrenePolymers

Introduction

Nutrient digestibility is a cornerstone of ruminant nutrition, directly impacting growth, milk production, and overall animal health; is fundamental to formulate and balance diets precisely, evaluating nutrient digestibility with the final objective to optimize feed efficiency and improving the health and productivity of livestock, while minimizing waste and environmental impact.

The term digestibility refers to the proportion of nutrients from feed that are absorbed and utilized by the animal rather than excreted as waste. In ruminants, nutrient absorption efficiency is affected by various physiological factors, including the structure and functionality of the digestive system, feed type and quality, nutrient composition, and feeding preferences between species (Harmon and Swanson, 2020; Nozière et al., 2010).

Researchers have developed several methods for assessing digestibility, ranging from *in vivo* methods, measured by total fecal collection, or determined indirectly by internal or external markers. Again, digestibility can be measured *in vitro* (Khan and Sarwar, 2003). Among these, internal markers like undigested neutral detergent fiber (uNDF), undigested neutral detergent fiber exclusive of residual ash (uNDFom), and acid insoluble ash (AIA) have become popular due to their ability to estimate digestibility without the need for complete fecal collection, which is labor-intensive and impractical on a large scale (Lee and Hristov, 2013).

As ruminants possess a multi-chambered stomach, accurately estimating the total tract digestibility of various nutrients becomes a key challenge. Considering the use of internal markers, the effectiveness of this process varies based on the type of diet and the specific characteristics of the animals, which can differ by feeding strategies (e.g. hay-based diets, silage-based diets, amount of concentrate) and nutritional preferences (e.g. browers, grazers). Therefore, it is of interest to understand if any internal marker could work for any ruminant species, or if, on

the contrary, there may be some internal markers which could perform better in estimating apparent and true total tract digestibility.

Overall, this work focuses on the comparative analysis of different internal markers— uNDF, uNDFom, and AIA as main ones, and occasional acid detergent lignin (ADL) and acid detergent insoluble ashed (ADIA) — for estimating totral tract apparent and true nutrient digestibility in small dairy ruminants, mediterranean dairy buffaloes, dairy and beef cattle.

Specifically, in Trial 1 we will focus onto the different estimations of ttaD and ttD of various nutrients in dairy small ruminants by comparing the total collection, uNDF, uNDFom and AIA.

In Trial 2, we will focus onto the different estimations of ttaD and ttD of various nutrients in dairy Mediterranean buffaloes by comparing uNDF and AIA.

In Trial 3, we will focus onto the different estimations of ttaD and ttD of various nutrients in dairy cows by comparing uNDF and uNDFom.

In Trial 4, we will focus onto the different estimations of ttaDMD, ttaCPD and ttNDFD in beef cattle by comparing uNDF, ADL and ADIA.

Furthermore, in Trial 5 this work will focus onto the practical implications of the use of markers in the assessment of nitrogen balance of dairy cows. Additionally, it includes two technical annexes (I and II) concerning specific analytical aspects of digestibility.

Marker-based digestibility estimations

Fiber fraction of plant that remains unfermented in the rumen and passes through the digestive system – uNDF - has been extensively studied as a marker for estimating total tract apparent digestibility (ttaD) and total tract digestibility (ttD) of various nutrients. Relatively recent advancements in analytical techniques, such as 240-hour fermentation trials, have allowed for more precise measurements of uNDF, making it a reliable marker for estimating fiber

digestibility (Raffrenato et al., 2018). However, questions remain regarding its accuracy across different species (Reis et al., 2017), fiber type and inclusion level in the diet, particularly in comparison to other markers like AIA and uNDFom.

The use uNDF exclusive of residual ash (uNDFom) offers an alternative to traditional uNDF markers by excluding residual ash from the calculation, providing a more refined estimate of fiber digestibility (Sirois, 2015). While uNDFom has been demonstrated to provide more accurate results in some cases, particularly in diets with lower ash content (Raffrenato and Van Amburgh, 2010), its broader application across various species and feed types is still under investigation. The performance of uNDFom, particularly in comparison to uNDF, is an important focus of this study.

Another historically widely used internal marker – AIA – has shown to be a promising in various ruminant digestibility studies, both for its short determination times and easiness of analysis (Sales and Janssens, 2003; Wang et al., 2020). Unlike uNDF, AIA is a non-digestible component of feed that remains unchanged as it passes through the gastrointestinal tract, with no difference reported in literature between species (Reis et al., 2017). Because of its stability and non-reactive nature, AIA is often considered a reliable marker for estimating ttaD and ttD. However, discrepancies in AIA recovery rates have been reported, with some studies indicating that it may underestimate digestibility in certain species or dietary conditions (Bergero et al., 2004).

Some authors indicated that AIA can be used to estimate ttaD and ttD when included in the diet at levels higher than 0.75% DM, with negligible circadian excretion differences (Thonney et al., 1985).

Importance of comparative marker analysis

One of the key challenges in digestibility research is the variability in marker performance depending on the type of animal, diet, and experimental conditions. For instance, dairy ruminants and beef cattle are typically fed diets with varying forage-to-concentrate ratios, which can impact the total content of markers, but should not impact their digestibility (Cotanch et al., 2017).Similarly, Mediterranean buffaloes, which are often fed silage-based diets, may exhibit different digestive efficiencies compared to small ruminants like sheep and goats, which are commonly fed hay-based diets and concentrate, separately (Guerra et al., 2024; Simoni et al., 2024).

Therefore, a comparative analysis of markers across different species and diet types is essential to question their limitations and identifying the most appropriate marker for each scenario, depending on animal species and forage type inclusion in the diet.

Previous research has highlighted both the strengths and weaknesses of these markers in different contexts. For example, uNDF has been shown to provide reliable estimates of fiber digestibility in dairy cows and beef cattle (Cotanch et al., 2017; Palmonari et al., 2016), but its accuracy may decrease when applied to small ruminants or buffaloes fed diets with high ash content (Reis et al., 2017). Similarly, AIA has been widely used in studies involving sheep and goats (Block et al., 1981; da Teixeira et al., 2018; Van Keulen and Young, 1977), but with extremely variable recovery rates (Lund et al., 2007), which raise questions about its suitability for all species.

On the other hand, uNDFom, has shown potential in providing more accurate estimates in high-fiber diets (Raffrenato et al., 2018), but its application in different practical feeding systems is still of interest.

This study aims to contribute to the markers study by analyzing the results of a series of trials that directly compare the performance of uNDF, uNDFom, and AIA, between other accessory markers like ADL and ADIA, across multiple ruminant species and diet types.

Practical application of makers for Nitrogen balance in dairy cows

Nitrogen balance (NB) is a crucial metric in dairy farming, reflecting the difference between the nitrogen (N) that cows assume and the N they excrete through milk, feces, and urine. A positive NB indicates higher N intake than N excreted, which can enhance milk production but may lead to increased N losses. This excess N can contribute to pollution of soil and water through runoff or leaching, potentially leading to ecological issues such as eutrophication (Tavernier et al., 2023). Conversely, a negative NB indicates N excretion higher than nitrogen intake, which points to inefficiencies in nitrogen utilization due to different potential reasons (e.g inadequate forms of supplementation). Ideally, a value of NB around zero should be ideal, considering having a sufficient amount of N intake to sustain production and health, but at the same time not allowing for high quantities of N in urines and feaces to be lost in the environment (Spanghero and Kowalski, 2021).

To accurately measure NB, reliable estimates of N excretion are essential. Fecal and urinary markers provide practical alternatives to total collection methods, making it feasible to assess NB in a variety of conditions in dairy farming field trials (Ferreira et al., 2023). For the total fecal output estimation, uNDF is often used, while for urinary N output, creatinine levels measured through spot sampling is a commonly used method. Creatinine levels in urine samples, combined with body weight estimates, provide an indirect measurement of total urine output (Valadares et al., 1999). However, the accuracy of this method can be influenced by factors such as the

handling of urine samples, including acidification to prevent nitrogen loss, which may affect creatinine concentrations (Danese et al., 2024).

Therefore, the second aim of this thesis is to analyze the results from a field trial on NB in dairy cows fed hay-based diets in the area of the Parmigiano Reggiano cheese production with a focus onto the use of markers to predict total fecal and urine output.

Trial 1: Comparison of mean values of total tract apparent and true digestibility calculated by total collection and estimated through uNDF, uNDFom and AIA in dairy small ruminants

Adapted and expanded from Simoni et al. (2024) and Danese et al. (2024).

Materials and methods

Samples collection and chemical analysis

For a complete description of the material and methods, please refer to the articles indicated above: briefly, the aim of Simoni et al. (2024) was to assess NIRs predictions of dairy sheep and goat's total tract digestibility fed alfalfa hay and concentrate at different ratios, while the second, still under review, to asses NIRs capacity to predict digestibility estimated by means of uNDF, uNDFom and AIA, in dairy sheep and goat's total tract digestibility, fed alfalfa hay and concentrate at different ratios.

A total of 195, 300 g individual fresh faecal samples were obtained from native Greek crossbreed dairy sheep, goats and rams from different trials conducted at the Department of Animal Science of the Agricultural University of Athens (Athens, Greece). The sheep and the goats were offered three diets with a forage (alfalfa) to concentrate ratio (F:C) of 40:60, 50:50 and 60:40. Whereas, the rams were fed only a 100% forage diet composed on average by 57% of alfalfa and 43% of straw. Samples of alfalfa, straw and concentrates were collected in each trial and chemically analyzed. Forages were supplied through basket hay feeders including a net to minimize sorting behavior. Forage and concentrates were fed separately once a day and faecal samples representative of the daily excretion were collected, after an adaptation period of at least 5 days. Individual forage and concentrate intake were measured by difference between the amounts administered and orts during the faecal sampling period. The intake was used to calculate the composition of the diet ingested by individual animals. The diets fed to the animals were evaluated by the Cornell Net Carbohydrate and Protein System for sheep and goats (Cannas et al., 2007) and they were found to cover the animals' nutritional requirements.

Both dietary ingredients and faecal samples were oven dried at 55 °C for 48 h and then ground in a Cyclotec mill (Tecator, Herndon, VA) to pass a 1-mm screen. For each sample an aliquot was chemically analysed, while another aliquot was subjected to spectrophotometric analysis. The chemical composition of the dietary ingredients and of the faecal samples was determined as follows: an aliquot of 5 g of pre-exsiccated sample was oven dried at 103 °C overnight to determine the DM content. Fibre fractions (NDF assayed with heat-stable amylase without sodium sulphite and expressed exclusive of residual ash – aNDFom; ADF expressed exclusive of residual ash – ADFom; and lignin) were analysed as described by Mertens et al. (2002). A repetition of the fibre fraction analysis was performed to collect the aNDF and ADF residues for fibre bound N determination. The CP content of each sample as well as the CP bound to aNDF (NDICP) and to ADF (ADICP) were determined by the combustion digestion of the sample at 900 °C in excess of oxygen by Dumatherm® (Gerhardt GmbH &Co, Königswinter, Germany) as described by Mihaljev et al. (2015). A further repetition of the fibre fractions analysis was performed sequentially for hemicellulose and cellulose determination (Robertson & Van Soest, 1981). Hemicellulose content was calculated as difference between aNDFom and ADFom, whereas cellulose content was calculated as difference between ADFom and lignin. The uNDF content was determined through a 240-h *in vitro* fermentation according to Raffrenato et al. (2018). Rumen fluid was collected at the slaughterhouse from 4 cows and processed as described in Simoni et al. (2020). Briefly, the rumen fluid kept at 39.5 °C under anaerobic conditions was blended and filtered through 4 layers of cheesecloths. Rumen fluid was inoculated at the ratio 1:4 to the medium in a flask containing 0.5 g of sample and incubated in an *in vitro* batch fermentation system (Goering and Van Soest, 1970). Ash was determined by combustion at 550°C for 4 h. The soluble CP was calculated as the difference between CP and NDICP.

Digestibility calculations and statistical analysis

The coefficient of total-tract apparent (tta) digestibility estimated (D) of DM (ttaDMD), crude protein (ttaCPD; CP), Soluble CP (ttaSolCPD) and coefficient of total-tract (tt) De of NDF (ttNDFD), ADF (ttADFD), hemicellulose (ttHemicelD), and cellulose (ttCelD) were calculated using the individual dietary uNDF, uNDFom or AIA assumed and the uNDF, uNDFom or AIA of the related faecal samples as described by Fustini et al. (2017) and Righi et al. (2017).

Statistical analyses were performed using Prism. Normality was assessed graphically and by using the Shapiro–Wilk test. Since digestibility of nutrient were not normally distributed, a Dunn's multiple comparison test was used to compare digestibility values for each nutrient between markers, *in vivo* by total collection and marker-estimated digestibility (total collection vs uNDF vs uNDFom vs AIA).

Linear regressions models were applied between uNDF, uNDFom or AIA (y; %DM) plotted against the TC (x; %DM), to evaluate differences in predictive ability of the markers employed, considering the estimation of ttaDMD, ttNDFD and ttaCPD. Furthermore, a Spearman's correlation coefficient was utilized to evaluate the relationship between TC and the before mentioned markers, used to estimate ttaDMD, ttNDFD and ttaCPD. Similarly, a linear regressions models were applied between excretion (y; g/day) plotted against its intake (x; g/day), for uNDF, uNDFom and AIA. lastly, a Sperman's correlation coefficient was utilized to evaluate the relationship between the intake and excreted undegradable materials, for uNDF, uNDFom and AIA.

Results and Discussion

Dietary and faecal chemical composition

The chemical composition of the dietary ingredients is reported in Table 1, whereas the calculated chemical compositions of the different diets consumed by the animals are shown in Table 2.

Table 1. Average chemical composition and standard deviation (±) of dietary ingredients.

Item[1]	Alfalfa			Wheat straw			Concentrate[2]		
Chemical composition, g/kg of DM unless otherwise indicated									
DM (g/kg)	918	±	23.0	922	±	19.0	913	±	48.0
Ash	82.8	±	12.4	69.8	±	11.3	56.0	±	9.00
CP	134	±	4.00	34.4	±	1.00	168	±	1.50
Ether extract	92.0	±	2.00	8.30	±	1.00	18.5	±	1.00
aNDF	513	±	52.0	743	±	21.0	193	±	10.6
NDICP	24.8	±	1.00	6.70	±	0.00	19.2	±	6.00
ADF	392	±	15.7	419	±	20.1	96.0	±	11.2
ADICP	8.8	±	3.00	8.60	±	1.00	54.1	±	2.00
Hemicel	121	±	20.4	324	±	19.1	97.1	±	3.70
Cel	321	±	18.3	382	±	6.40	66.1	±	11.6
Lignin	63.7	±	21.6	29.4	±	5.80	22.3	±	16.3
Starch	-		-	-		-	412	±	1.00
uNDFom	319	±	71.0	285	±	28.2	69.3	±	4.00
AIA	37.9	±	13.8	39.7	±	18.0	20.0	±	1.90

[1]DM= DM of pre-dried samples; aNDF= amylase treated NDF with residual ash; NDICP= protein bound to NDF; ADF= ADF with residual ash; ADICP= protein bound to ADF; uNDFom= undigestible NDF evaluated after 240-h of fermentation and expressed as exclusive or residual ash, AIA= acid insoluble ashes.

[2]Concentrate ingredients: maize grain 45.2%, wheat middling 15%, sunflower meal 8%, soybean meal 15%, barley grain 12.5%, mineral and vitamin complex 2%, calcium phosphate 1.5%, calcium carconate 0.5%, sodium chloride 0.3%.

Table 2. Calculated chemical composition of the diets assumed by the small ruminants offered 4 different forage to concentrate ratio (F:C; 40:60, 50:50 and 60:40 alfalfa hay and concentrate respectively) or 100 % forage (43% alfalfa and 57% straw).

F:C offered	40:60	50:50	60:40	100:00
Dietary Item (g/kg of DM)[1]				
Ash	53 ± 0.4	54 ± 0.5	56 ± 0.5	59 ± 0.7
CP	130 ± 0.6	128 ± 0.9	125 ± 0.8	78 ± 6.1
Ether extract	15 ± 0.2	14 ± 0.3	13 ± 0.3	9 ± 0.1
aNDFom	325 ± 7.2	352 ± 10.2	382 ± 9.5	609 ± 16.5
NDICP	21 ± 0.1	22 ± 0.2	22 ± 0.2	17 ± 1.3
ADFom	212 ± 6.4	237 ± 9.1	263 ± 8.5	394 ± 2.1
ADIN	41 ± 0.7	39 ± 1.0	36 ± 1.0	17 ± 1.0
Hemicel	113 ± 0.8	115 ± 1.1	119 ± 1.0	215 ± 14.4
Cel	188 ± 5.4	209 ± 7.6	231 ± 7.1	352 ± 3.7
Lignin	24 ± 1.0	28 ± 1.5	32 ± 1.4	42 ± 1.6
Starch	245 ± 9.3	209 ± 13.1	171 ± 12.3	- ± -
uNDFom	169 5.7	191 8.0	214 7.5	304 2.4
AIA	27.2 ± 1.2	29.1 ± 1.2	30.9 ± 1.2	39.8 ± 0

[1]DM= DM of pre-dried samples; aNDFom= amylase treated NDF without residual ash; NDICP= protein bound to NDF; ADFom= ADF without residual ash; ADICP= protein bound to ADF; uNDFom= undigestible NDF evaluated after 240-h of fermentation and expressed as exclusive or residual ash.

Averagely, all the parameters are to be considered withing literature ranges, as described in Table 3, and discussed in the following paragraph. Whether we considered DM, Ash, CP or NDF content, the variability range between authors is notably wide: small ruminants are selective browsers and grazers, therefore changing their preferences from time to time, depending on the environment and season considered, from roughages to extremely fresh sprouts. This results in an animal with highly variable nutritional preferences, ranging from forages characterized by

extreme lignification, slow passage rates, low soluble crude protein (CP), and sugars to those with completely opposite attributes: feaces will be the mirror of the animals' preferences. Therefore, it is unprecise, and beyond scope for this chapter, thoughtfully confront the feacal composition of our trial with the ones reported in literature. Briefly, with a 56.3 % of humidity, feaces had an ash content averaging around 12.7% DM. The mean faecal CP content was ranged between 11.7 to 15.4 % DM, with from 69.4 to 75.1 % CP as soluble CP. Faecal aNDF content ranged from 58.0 to 65.7% DM, with about 2/3 being ADF. Hemicellulose content varied between 16.2 and 20.9 % DM, cellulose between 14.6 and 29.2 % DM, and ADL from 12.5 to 41.3 % DM. The uNDFom content ranged from 44.4 to 54.1 % DM and AIA from 0.43 to 1.13 % DM. For a complete description, please refer to Simoni et al. (2024).

Lyons et al. (2016) reported wide ranges of DM content from 150 g/kg for lowland grass to 895 g/kg for grass nuts-fed sheep. Following the same line, the same author and other colleagues reported faecal ash ranges going from 80 g/kg up to 220 g/kg (Jalali et al., 2012b; Lyons et al., 2016).Also, CP values fall withing another authors' range, with values ranging from 83 to 247 g/kg (Kyvsgaard et al., 2000). Fibre fractions, as NDF, ADF, Hemicellulose and cellulose are withing ranges reported in literature (Lyons et al., 2016; Sales and Janssens, 2003), while our fecal lignin are higher than those report by the first latter author.

Table 3. Chemical composition (g/kg DM) of small ruminants pre-exsiccated faeces (n = 195).

Item[1]	Mean		SD
Chemical composition, g/kg of DM			
DM, g/kg	929	±	8
Ash	127	±	19.6
CP	136	±	18.5
aNDF	619	±	38.6
NDICP	36.6	±	5.30
ADF	433	±	30.9
ADICP	20.7	±	3.40
Hemicellulose	186	±	23.5
Cellulose	219	±	73.0
Lignin	214	±	69.8
uNDFom	493	±	48.9
AIA	7.81	±	3.48

[1]DM= DM of pre-dried samples; aNDFom= amylase treated NDF without residual ash; NDICP= protein bound to NDF; ADFom= ADF without residual ash; ADICP= protein bound to ADF; uNDFom= undigestible NDF evaluated after 240-h of fermentation and expressed as exclusive or residual ash, AIA= acid insoluble ashes.

Total-tract apparent and true digestibility estimated through uNDF, uNDFom and AIA, compared to total faecal collection

The descriptive statistics of total tract apparent digestibility (tta) and total tract digestibility (tt) using uNDF, uNDFom and AIA respectively, are reported in Tables 4, 5, and 6. When uNDF was used as an internal marker, average values for ttaDMD, ttCelluloseD, and ttaSolubleCPD exceeded 627 g/kg, with ttaCPD averaging 584 g/kg. In contrast, all other parameters fell below 500 g/kg, with variability ranging from 9% for ttaDMD to 59.4% for ttaAshD. Similarly, using uNDFom as a marker, ttaDMD, ttCelluloseD, and ttSolubleCPD were all above 604 g/kg, while ttaCPD averaged 562 g/kg. Other parameters stayed below 402 g/kg, with a variability range between 11% for ttaDMD and 67.7% for ttaAshD. When AIA was used, values peaked at 588 g/kg for ttSolubleCPD, followed closely by ttDMD, ttCelD, and ttCPD. All other parameters were lower than ttAshD (239 g/kg), with variability spanning from 24.7% for ttaSolCPD to 71.1% for ttaAshD

Table 4. Descriptive statistics of average total-tract apparent (tta) and total-tract true (tt) of nutrients digestibility estimated (D) using faecal uNDF as internal marker (g/kg).

Trait[1]	Mean	SD[2]	Minimum	Maximum	CV[3]
ttaDMD	630	58.2	464	742	92.2
ttNDFD	376	58.4	147	503	155
ttADFD	346	67,5	110	470	195
ttHemicelD	433	67.3	216	612	155
ttCelD	627	128	408	955	205
ttaCPD	584	96,5	111	722	165
ttaSolCPD	646	58.4	459	774	90.5
ttaAshD	195	116	4.02	466	594
pdNDFD	754	108	369	985	144

[1]ttaDMD=total tract apparent dry matter digestibility; ttNDFD=total tract amylase treated neutral detergent fibre; ttADFD=total tract acid detergent fibre digestibility; ttHemicelD=total tract hemicellulose digestibility; ttCelD=total tract cellulose digestibility; ttaCPD=total tract apparent crude protein digestibility; ttaSolCPD=total tract apparent soluble crude protein digestibility; ttaAshD=total tract apparent ash digestibility, ttpdNDFD= total tract amylase treated potentially degradable neutral detergent fibre.
[2]SD=standard deviation.
[3]CV=coefficient of variation.

Table 5. Descriptive statistics of average total-tract apparent (tta) and total-tract true (tt) of nutrients digestibility estimated (D) using faecal uNDFom as internal marker.

Trait[1]	Mean	SD[2]	Minimum	Maximum	CV[3]
ttaDMD	604	66.5	315	717	110
ttNDFD	340	51.2	110	458	151
ttADFD	310	54.6	109	423	176
ttHemicelD	402	71.4	221	782	177
ttCelD	606	133	401	954	219
ttaCPD	562	95.0	80.9	708	169
ttaSolCPD	613	82.0	222	751	134
ttaAshD	174	118	4,83	849	677
ttpdNDFD	735	7.80	251	957	148

[1]ttaDMD=total tract apparent dry matter digestibility; ttNDFD=total tract amylase treated neutral detergent fibre; ttADFD=total tract acid detergent fibre digestibility; ttHemicelD=total tract hemicellulose digestibility; ttCelD=total tract cellulose digestibility; ttaCPD=total tract apparent crude protein digestibility; ttaSolCPD=total tract apparent soluble crude protein digestibility; ttaAshD=total tract apparent ash digestibility; ttpdNDFD= total tract amylase treated potentially degradable neutral detergent fibre.
[2]SD=standard deviation.
[3]CV=coefficient of variation.

Table 6. Descriptive statistics of average total-tract apparent (tta) and total-tract true (tt) of nutrients digestibility estimated (D) using faecal AIA as internal marker.

Trait[1]	Mean	SD[2]	Minimum	Maximum	CV[3]
ttaDMD	586	145	155	916	247
ttNDFD	361	175	28.5	854	484
ttADFD	337	184	3.12	844	545
ttHemicelD	393	184	3.77	873	468
ttCelD	565	217	21.9	955	383
ttaCPD	535	163	64.2	902	305
ttaSolCPD	588	147	153	915	250
ttaAshD	239	170	0.55	843	711
ttpdNDFD	723	146	273	976	201

[1]ttaDMD=total tract apparent dry matter digestibility; ttNDFD=total tract amylase treated neutral detergent fibre; ttADFD=total tract acid detergent fibre digestibility; ttHemicelD=total tract hemicellulose digestibility; ttCelD=total tract cellulose digestibility; ttaCPD=total tract apparent crude protein digestibility; ttaSolCPD=total tract apparent soluble crude protein digestibility; ttaAshD=total tract apparent ash digestibility; ttpdNDFD= total tract amylase treated potentially degradable neutral detergent fibre.
[2]SD=standard deviation.
[3]CV=coefficient of variation.

Feacal digestibility markers have been extensively employed and studies in small ruminant nutrition: while the use of uNDF or uNDFom as marker to total tract apparent digestibility is still debated (Reis et al., 2017), AIA has been more commonly employed (Sales and Janssens, 2003). Together with several well studied markers, the undegradable DM (uDM) has already been used in the past to estimate ttaDMD in sheep by Fondevila et al. (1995). These authors found correlation coefficients of 0.793 and 0.806 between total collection (TC) and ttaDMD estimated using uDM and uNDF, respectively: the use of uDM overestimated ttaDMD (+2.5%), while the use of uNDF underestimated the same parameter (-2.2%), compared to TC.

On a similar trend, our results confirms that ttaDMD, estimated with uNDF, can lead to and slight underestimation (-6.82%), compared to TC. Another author (Kahdem et al., 2007) also used the TC method in sheep, which differed by -3.87 %, with our TC results (66.88 vs 70.75 %).

Table 7 presents a comparison of ttaD and ttD by TC, and estimated using uNDF, uNDFom, and AIA, across various digestibility parameters. Statistical differences between the TC and markers is evident, with uNDF consistently showing numerical higher digestibility estimates compared to both uNDFom and AIA, but still statistically different from TC results. For example, in terms of ttaDMD, uNDF provides the closer numerical digestibility value (63.08%) compared to TC, followed closely by uNDFom (60.97%), while AIA gives a significantly lower estimate (58.61%). The same trend is observed for ttNDFD, where uNDF records the higher numerical value (37.27%), with uNDFom (33.92%) and AIA (35.73%) showing lower digestibility estimations.

As reported by Simoni et al., (2024), an outdated work by Gihad et al. (1980), focusing on apparent digestibility of Zambian natural grass hay fed to goats by means of only external markers, reported comparable values of ttaDMD using uNDF (+ 0.6 %), uDNFom (- 3.2 %) and AIA (+ 5%).

Conversely, the overall ttaDMD estimation from the present study, considering all the three markers employed (uNDF, uNDFom, and AIA; 58 – 63 %) is not comparable with the range reported by Fondevila et al. (1995; 41 – 59 %), probably due to their use of uDM as internal predicting marker, which can lead to substantial numerical differences in the estimation of ttaDMD.

In a study conducted by Luginbuhl et al. (2000), where a similar diet was administered to a control group of goats, digestibility of cellulose and hemicellulose calculated by total faecal collection resulted in higher values compared to what found in the present trial using uNDF, uNDFom and AIA, respectively (ttHemicelD + 20.9, 24 and 24.9 % and ttCelD + 16.7, 18.8 and 22.8 %).

Overall, our results are in partial contrast with what reported in Danese et al. (2024; paper under revision), where considering solely sheep and goats, and not comprehending rams, AIA were found to give numerically closer estimates of ttaD and ttD, but still statistically different, compared to uNDF and uNDFom. It appears that, including rams fed solely with straw, which contains less AIA compared to alfalfa and concentrate, estimated values of tta and ttaD using AIA numerically decrease compared to uNDF and uNDFom. This poses a widely discusses question on the use of markers to estimate ttaD and ttD, since is once more proven that differing the dietary inclusions, one marker could overperform others previously considered better, compared to TC, as in this case of uNDF and AIA.

Table 7. Comparison of the descriptive statistics of average total-tract apparent (tta) and total-tract (tt) nutrients digestibility (D) by total collection (TC) and estimated using faecal uNDF, uNDFom and AIA as a marker of pre-exsiccated faeces of small ruminants.

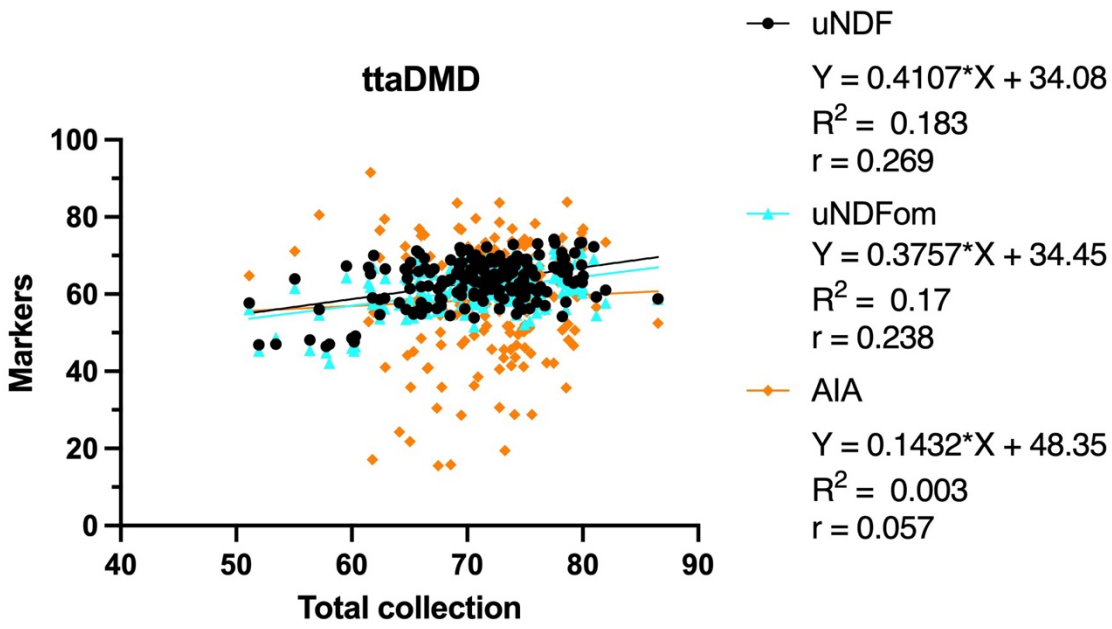
			Estimated digestibilty with markers							
	TC		uNDF		uNDFom		AIA		SEM	p-Value
ttaDMD	70.75	a	63.08	b	60.97	c	58.61	bc	0.5425	<0.0001
ttNDFD	50.33	a	37.40	b	33.98	c	36.14	b	0.7588	<0.0001
ttNDICP	50.27	a	39.29	b	35.61	c	36.60	bc	0.9191	<0.0001
ttADFD	48.07	a	34.69	b	31.07	c	33.61	b	0.8126	<0.0001
ttADICP	84.55	a	80.30	b	79.34	b	78.02	b	0.3956	<0.0001
ttHemicelD	55.11	a	42.68	b	39.46	c	38.34	bc	0.7875	<0.0001
ttCelD	70.25	a	62.52	b	60.42	b	55.94	b	1.1170	<0.0001
ttaCPD	68.35	a	60.50	b	58.12	c	54.79	bc	0.6152	<0.0001
ttaSolCPD	71.83	a	64.92	b	62.81	c	59.81	bc	0.5747	<0.0001
ttaAshD	31.20	a	19.82	bc	17.00	c	24.00	b	1.1270	<0.0001
ttpdNDF	80.96	a	75.35	b	74.12	b	71.78	b	0.8265	<0.0001

[1]ttaDMD=total tract apparent dry matter digestibility; ttaAshD=total tract apparent ash digestibility; ttaCPD=total tract apparent crude protein digestibility; ttaSolCPD=total tract apparent soluble crude protein digestibility; ttNDFD=total tract amylase treated neutral detergent fibre; ttNDICPD=total tract neutral detergent insoluble crude protein digestibility; ttADFD=total tract acid detergent fibre digestibility; ttADICPD=total tract acid detergent insoluble crude protein digestibility; ttHemicelD=total tract hemicellulose digestibility; ttCelD=total tract cellulose digestibility; ttpdNDFD=total tract potentially degradable amylase treated neutral detergent fibre.
[2](a-d) Different lowercase letter within a row are statistically different ($p<0.001$).

To further evaluate this aspect, in Figures 1, 2 and 3 are reported the regression and correlation coefficients of the makers used (y; uNDF, uNDFom and AIA, %DM) plotted with TC (x; %DM), for ttaDMD, ttNDFD and ttaCPD.

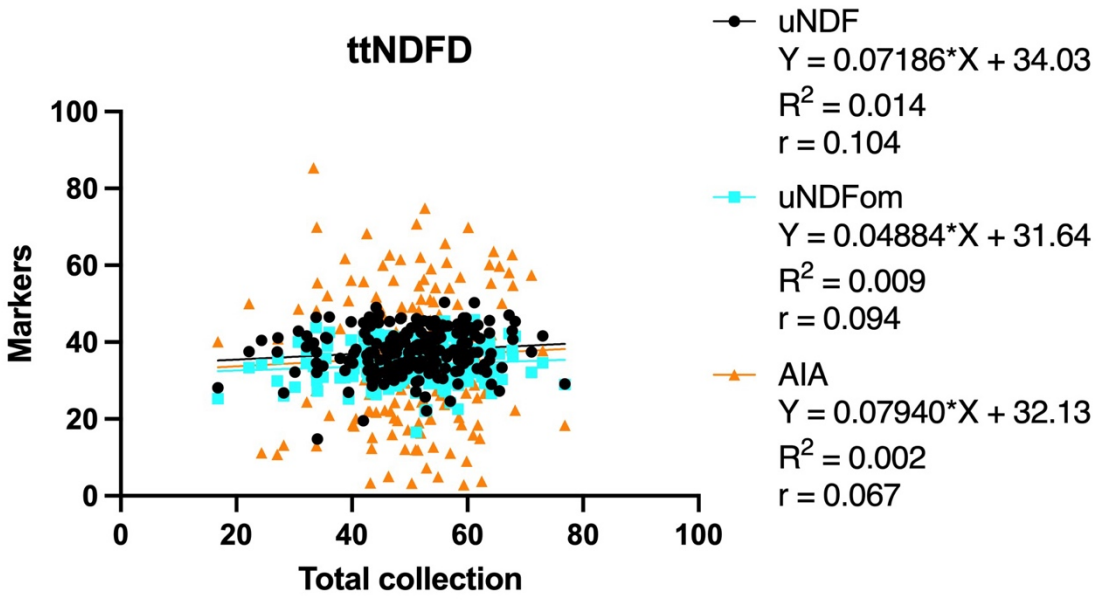
Considering ttaDMD, reported in Figure 1, uNDF and uNDFom seemed to perform better ($R^2 = 0.183$ and 0.170) compared to AIA ($R^2 = 0.003$), confirmed also by the same trend maintained by the slopes of the three equations, similarly to the Spearman correlation coefficients.

Figure 1. Graphical representation of the linear regression between different markers (y; uNDF, uNDFom and AIA, %DM) plotted against TC (x; % DM), for ttaDMD.



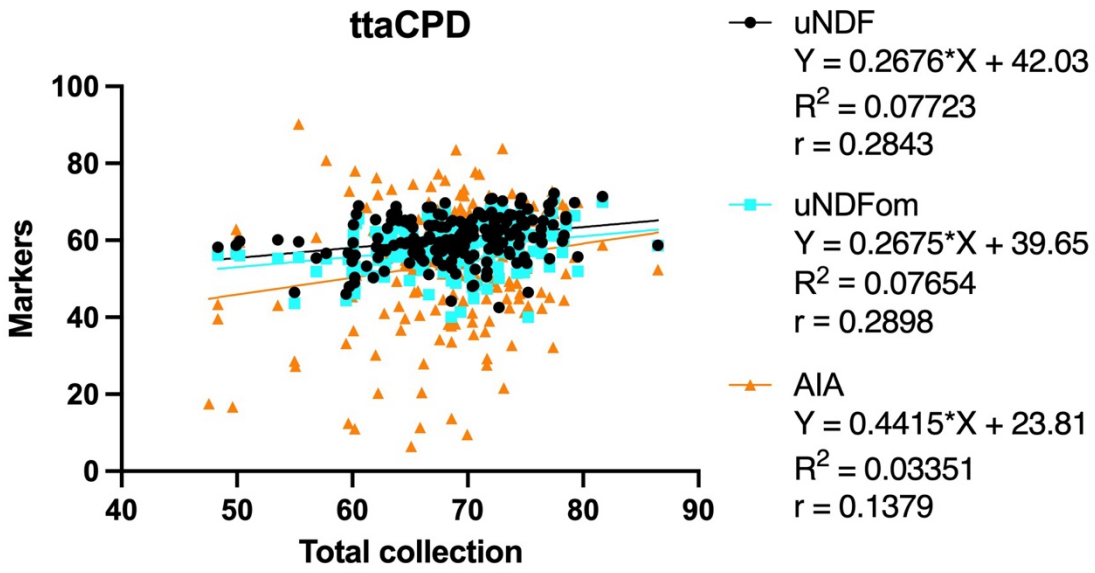
Considering ttNDFD, uNDF and uNDFom seems to perform better (R^2 = 0.014 and 0.009) compared to AIA (R^2 = 0.002); this trend is not confirmed here by the slopes of the three equations, where AIA maintains an higher slope compared to the other markers. The Spearman correlation coefficients follow the decreasing trend, from uNDF, uNDFom to AIA (0.104, 0.094 to 0.067).

Figure 2. Graphical representation of the linear regression between different markers (y; uNDF, uNDFom and AIA, %DM) plotted against TC (x; % DM), for ttNDFD.



Evaluating ttaCPD, the regression and correlation coefficients moves similarly to what reported to ttNDFD, where uNDF and uNDFom performed better than AIA (R^2 = 0.07, 0.07 vs 0.03).

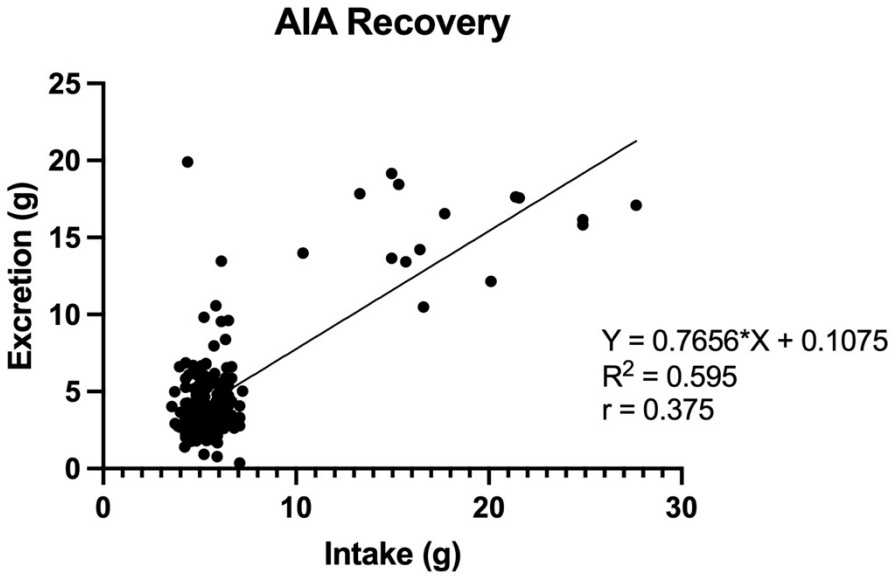
Figure 3. Graphical representation of the linear regression between different markers (y; uNDF, uNDFom and AIA, %DM) plotted against TC (x; % DM), for ttaCPD.



Recovery of the markers

The comparison of uNDF, uNDFom, and AIA as markers highlights distinct differences in their relationship between intake and excretion, as summarized in Table 7. In Figure 4, AIA displays the highest predictive power, with an R^2 value of 0.595 and a slope of 0.765, suggesting a robust response to intake, where excretion rises proportionally and significantly with intake. However, AIA's lower correlation coefficient (r = 0.357) indicates variability that may not be fully captured by its predictive strength alone.

Figure 4. Graphical representation of the linear regression of the recovery, as excretion (y; g/day) plotted against its intake (x; g/day), for AIA.



In contrast, uNDF and uNDFom, with moderate R^2 values of 0.268 and 0.311 respectively (Figure 5 and 6), show stronger correlation coefficients (r = 0.522 and 0.566, respectively). This implies that, although uNDF and uNDFom have lower predictive power, they demonstrate a more consistent intake-excretion relationship, suggesting they may provide a more reliable estimate of digestibility. Thus, while AIA may excel in predicting excretion based on intake, its variability may limit its utility, whereas uNDFom, balancing moderate predictive power with stronger correlation, could offer a more stable and reliable estimation. These results need to be evaluated also considering marker excretion patterns and the protocol applied (in this case, one representative daily fecal sample): uNDF and uNDFom are reported to have a circadian excretion pattern, while AIA is reported to have a less variable excretion pattern (Thonney et al., 1985). As we reported, uNDF and uNDFom have a more consistent intake-excretion relationship than AIA, but a lower predictive power. This could be due to their evaluation in a context of single daily sampling, which could probably not capture entirely the circadian pattern excretion.

Figure 5. Graphical representation of the linear regression of the recovery, as excretion (y; g/day) plotted against its intake (x; g/day), for uNDF.

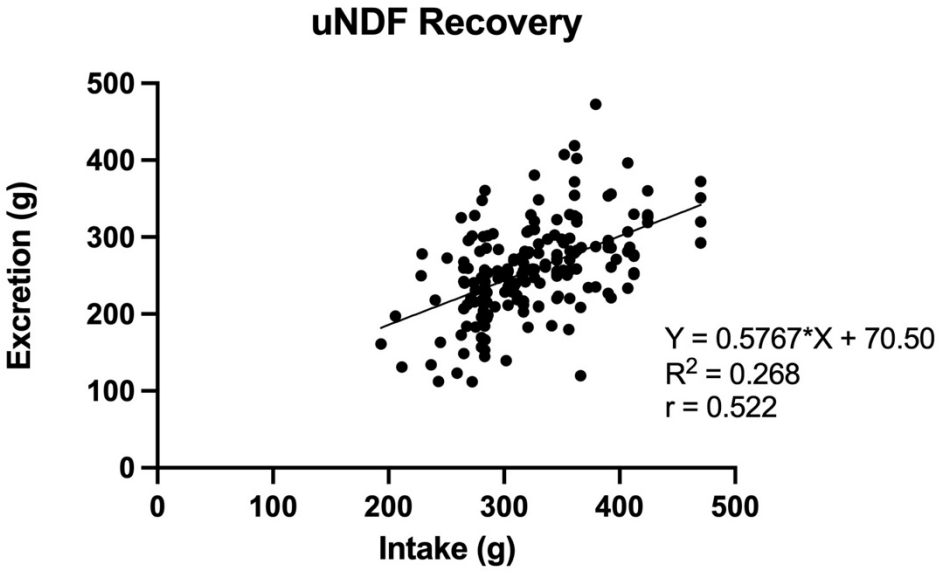
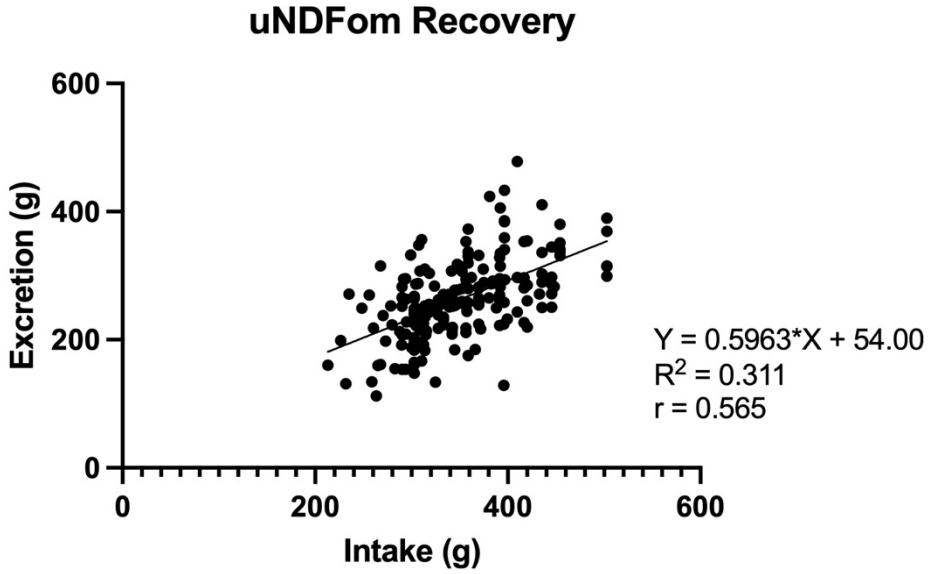


Figure 6. Graphical representation of the linear regression of the recovery, as excretion (y; g/day) plotted against its intake (x; g/day), for uNDF.



Consistent with these findings, several studies have reported lower correlation between intake and excretion of AIA, compared to uNDF (Sales and Janssens, 2003). De Carvalho et al. (2013) found that in digestibility trials with sheep and goats, indigestible dry matter (iDM) and iNDF exhibited complete fecal recovery, confirming their accuracy as markers for digestibility coefficients and fecal

excretion. However, indigestible iADF, which could be chemically comparable to AIA (Sunvold and Cochran, 1991), showed significant long-term bias with recovery rates of -9.12% in sheep and -3.02% in goats. Therefore, while AIA might show high predictive power, uNDF and uNDFom offer more reliable estimations, particularly in studies where stability and consistency are key considerations.

Table 7. Regression and correlation coefficients of the recovery, as excretion (y; g/day) plotted against its intake (x; g/day), for uNDF, uNDFom and AIA.

		Regression		Correlation
Marker	R^2	Intercept	Slope; p	r; p
uNDF	0.268	70.5	0.576; <0.0001	0.522; <0.0001
uNDFom	0.311	54.0	0.596; <0.0001	0.565; <0.0001
AIA	0.595	0.10	0.765; <0.0001	0.357; <0.0001

Conclusions

In conclusion, the study demonstrated significant variations in ttaD and ttD values depending on the internal markers used (uNDF, uNDFom, and AIA) across different nutritional components, in dairy small ruminants fed different forage to concentrate ratios. Our findings suggest that uNDF consistently provided less variable, numerically higher, and statistically different digestibility estimates compared to AIA, particularly in parameters such as ttaDMD. Although AIA has been widely used in digestibility studies, our results suggests that it potentially underestimate digestibility, possibly due to its incomplete recovery in fecal samples: therefore, its use shall be considered also based on the dietary content of AIA and the specific animal nutritional strategies, specifically if not in an experimental/research environment.

In contrast, uNDF showed more reliable recovery, showing a potential higher accuracy in estimating digestibility. Overall, variability in marker performance highlights the importance of selecting appropriate markers based on specific dietary compositions and animal species to ensure more accurate results in digestibility trials.

Trial 2. Comparison of mean values of total tract apparent and true digestibility estimated through uNDF and AIA in Mediterranean dairy buffaloes

Adapted from Guerra et al. (2024).

Materials and Methods

Samples collection and chemical analysis

For a detailed and complete description of the trial's material and methods please refer to Guerra et al. (2024).

Briefly, a total of 147 fresh faecal samples were collected from lactating buffaloes (155.8 ± 57.4 days in milk, 3.1 ± 2.2 parity, 10.5 ± 2.6 kg milk yield) across five farms in the Mozzarella di Bufala Campana PDO region. The farms were chosen for their dietary variation, with forage-to-concentrate ratios ranging from 25:75 to 52:48. All buffaloes were fed a silage-based total mixed ration (TMR) once daily, consisting of corn silage, alfalfa hay, haylage, mixed hay, concentrates, and a mineral–vitamin supplement, ensuring similar and comparable chemical and nutritional profiles. Dietary samples were collected over two days before faecal collection. Post TMR collection, the samples were stored at −20°C at the University Federico II (Naples, Italy), and later analyzed at the University of Parma (Parma, Italy).

Approximately 2 kg of feces was collected from each animal's rectum about 3 hours after feeding. Both TMR and faecal samples were oven-dried at 55°C until they reached a constant weight, then ground using a Retch SK mill (Bauknecht, Stuttgart, Germany) to pass through a 1-mm screen. Dry matter (DM), ash, and ether extract (EE) were analyzed according to European Commission Regulation No. 152/2009. Amylase-treated neutral detergent fiber without residual ash (aNDFom), acid detergent fiber without residual ash (ADFom), and acid detergent lignin (ADL) were analyzed using heat-stable amylase and corrected for ash (Mertens et al., 2002). Duplicate analyses were conducted, with additional repetitions for hemicellulose (Hemicel) and cellulose (Cel) determination, calculated sequentially as differences between aNDF and ADF, and between ADF and ADL, respectively. Nitrogen (N), neutral detergent insoluble nitrogen (NDIN), and acid detergent insoluble nitrogen (ADIN) were measured by combustion digestion at 900°C in excess oxygen using a Dumatherm (Gerhardt GmbH & Co, Königswinter, Germany) as described by

Mihaljev et al. (2015). Starch content was determined enzymatically (method 2014.10; AOAC International, 2014). Acid-insoluble ash (AIA) was analyzed on 5 g samples, burned, boiled with 2N HCl for 15 minutes (Bergero et al., 2009), filtered (Whatman no. 41), and ashed at 550°C per European Commission Regulation No. 152/2009. The undigested neutral detergent fiber (uNDF) content was determined through 240-hour fermentation (Raffrenato et al., 2018) in an in vitro batch system, using rumen fluid from four cows, as processed by Simoni et al. (2020).

Digestibility Calculations and statistical analysis

The estimated apparent total-tract (tta) digestibility (D) of DM (ttaDMD), OM (ttaOMD), ash (ttaAsh), EE (ttaEED), crude protein (ttaCPD; CP), Starch (ttaStarchD) and total-tract (tt) D of aNDFom (ttaNDFomD), ADFom (ttADFomD), Hemicellulose (ttHemicelD), and Cellulose (ttCelD), NDIN (ttNDIND), ADIN (ttADIND) were calculated using both uNDF and the AIA of the TMR and of the related faecal samples as internal markers as described by Fustini et al. (2017). Statistical analyses were performed using Prism. Normality was assessed graphically and by using the Shapiro–Wilk test. Since digestibility of nutrient were not normally distributed, a Mann-Whitney test was used to compare digestibility values for each nutrient between markers, total collection and marker-estimated digestibility (uNDF vs AIA). Linear regressions models were applied between uNDF (y; %DM) plotted against AIA (x; %DM), to evaluate the relationship between the predictive abilities of the two markers, considering the estimation of ttaDMD, ttNDFD and ttaCPD. Furthermore, a Sperman's correlation coefficient was applied to evaluate the relationship between uNDF and AIA, used to estimate ttaDMD, ttNDFD and ttaCPD.

Results and Discussion

Dietary and faecal chemical composition

Descriptive statistic of the dietary chemical composition is described in Table 1.

Table 1. Average chemical composition (% DM) of the diet fed to the lactating buffaloes.

Item	mean	SD[1]	min	max	CV[2]
Ingredients					
Mix silage (corn; wheat)	31.3	8.9	10.2	32.0	43.9
Mixed hay	25.6	1.8	10.3	16.1	14.0
Straw	10.1	7.0	5.1	15.1	69.6
Rye grass	15.3	-	15.3	15.3	-
Alfalfa haylage	8.3	1.8	7.1	9.5	21.1
Brewers grain	50.3	2.5	47.5	52.2	4.9
Earlage	14.5	-	14.5	14.5	-
Corn meal	14.4	-	14.4	14.4	-
Flaked corn	4.9	1.9	3.5	6.2	38.2
Soybean meal	5.6	4.3	2.0	10.4	78.0
Feedstuff	15.9	12.9	6.2	36.8	91.4
Minerals and vitamins	0.6	0.2	0.4	0.9	33.9
Chemical composition[3]					
DM, % as fed	44.3	7.7	39.0	57.1	17.4
OM	92.4	1.2	90.5	93.6	1.3
Ash	8.0	1.2	6.7	9.4	15.1
EE	5.2	2.0	2.8	7.0	39.1
aNDFom	42.9	3.0	38.1	45.9	6.9
ADFom	25.6	3.6	23.4	32.0	14.1
ADL	4.8	0.6	4.0	5.7	13.3
Hemicellulose	16.7	3.4	12.3	21.5	20.3
Cellulose	21.4	1.2	19.9	23.0	5.6
CP	14.5	1.0	13.4	16.0	7.0
NDIN	0.5	0.2	0.3	0.8	34.7
ADIN	0.2	0.1	0.1	0.3	28.2
Starch	21.7	3.1	16.9	25.8	14.1
AIA	1.8	0.3	1.4	2.4	18.1
uNDF	14.7	1.5	12.4	16.5	10.4

[1]SD=standard deviation.
[2]CV=coefficient of variation (%).
[3]DM= DM of pre-dried samples; OM= OM of pre-dried samples; EE= ether extract; aNDFom= amylase treated NDF without residual ash; ADFom= ADF without residual ash; NDIN= N bound to NDF; ADIN= N bound to ADF; AIA=acid insoluble ashes; uNDF= undigestible NDF evaluated after 240-h of fermentation; ADIN= N bound to ADF; AIA=acid insoluble ashes; uNDF= undigestible NDF evaluated after 240-h of fermentation.

Table 2. Average chemical composition (% DM) of the faeces of the lactating buffaloes (n=147).

Chemical composition[1]	mean	SD[2]	min	max	CV [3]
DM, % of predried samples	934	7	915	964	7
OM	845	29	753	917	34
Ash	154	27	104	247	178
EE	19	5	9	38	244
aNDFom	575	47	404	687	82
aNDF	621	47	452	715	76
ADFom	361	33	272	436	92
ADF	399	34	272	47	85
ADL	98	15	31	155	15
Hemicel	242	37	107	386	11
Cel	279	38	152	376	137
CP	142	13	111	175	91
NDIN	8	1	5	13	143
ADIN	5	1	3	08	18
Starch	17	6	3	56	374
AIA	61	10	39	81	17
uNDF	414	57	276	601	137

[1]DM= DM of pre-dried samples; OM= OM of pre-dried samples; EE= ether extract; aNDFom= amylase treated NDF without residual ash; ADFom= ADF without residual ash; NDIN= N bound to NDF; ADIN= N bound to ADF; AIA=acid insoluble ashes; uNDF= undigestible NDF evaluated after 240-h of fermentation; ADIN= N bound to ADF; AIA=acid insoluble ashes; uNDF= undigestible NDF evaluated after 240-h of fermentation.
[2]SD=standard deviation.
[3]CV=coefficient of variation (%).

The fecal composition, as described in Table 2, exhibited significant variability, likely influenced by dietary differences and individual animal factors. CV for fecal components was generally high, exceeding 12%, except for DM, aNDFom, and N, which had CV values of 0.7%, 8.2%, and 9.1%, respectively. Fecal aNDFom content averaged 57.5% of DM, with 62.8% as ADFom and 17% as lignin, highlighting the portion of the diet's fiber that remains undigested. Fecal CP content was 14.2% of DM, while NDIN ranged from 0.5 to 1.3 and ADIN from 0.3 to 0.8 % DM. The dietary uNDF content averaged 14.7 % of DM, while fecal uNDF was markedly higher at 41.3% DM, resulting in a difference of + 26.6%. Similarly, dietary AIA was 1.8% of DM, compared to a fecal AIA content of 6.08% of DM, leading to a difference of + 4.28%.

The DM content of feces observed in our study (15.4% ± 3.8%, as-is) is consistent with values reported by Bovera et al. (2007), similarto the percentages of ash, EE, aNDFom, and ADFom also reported by the latter author. The ADFom, Cel, and ADL contents in our study are comparable to those reported for domestic buffalo by Lisanti et al. (2021). The N content of buffalo feces in our study ranged from 1.78% to 2.80% DM, which is lower than the values reported for buffalo heifers by Al-Asfoor et al. (2013). This discrepancy may be attributed to the higher N requirements and supply in lactating animals compared to heifers. Comparing to beef faecal content, the NDIN and ADIN values in buffalo feces were similar with what reported by Simoni et al. (2021). Furthermore, the fecal starch content observed (average 1.66% of DM) was lower than the values reported for male, lactating, and dry Mediterranean buffaloes by Bovera et al. (2007) and Paula et al. (2020). The minimum fecal starch value recorded (0.25% of DM) is close to the average value found in heifer feces as reported by Al-Asfoor et al. (2012): this lower faecal starch content might be due to more efficient starch digestion in lactating animals or variations in dietary starch sources, a hypothesis supported by Grant and Ferraretto (2018). Righi et al. (2007) also provide relevant data about this topic, showing similar starch digestion efficiency in lactating cows fed a diet with an average starch content of 18.1% of DM, comparable to the diet in our study, in which fecal starch proportions ranged from 0.11% to 2.75% DM.

Internal marker concentration, starting from AIA, was consistent with the study by Thonney et al. (1985), where the fecal AIA in steers fed a corn silage-based diet was on average 3.6 times higher than the dietary AIA, compared to our values of 3.4 times. In contrast, in small ruminants fed hay-based diets, the fecal AIA was on average 1.2 times higher than the dietary AIA, as found by Simoni et al. (2024). These findings emphasize the significant, but often overlooked, impact that differences in diet composition and digestibility, feeding management, and species-specific preferences can have on the efficacy of AIA as a dietary marker.

In our study, fecal uNDF averaged 41.3% DM, starting from a dietary concentration of 14.6% DM. This finding aligns with the study on Murrah buffaloes by Soares et al. (2011), which reported a

dietary uNDF content of approximately 36% DM, leading to an estimated fecal uNDF content of 61% DM. The dietary to faecal uNDF ratio in our study ranged from 2.71 to 6.20, indicating lower dietary fiber digestibility in buffalo compared to dairy cows (1.58–4.10) as reported by Righi et al. (2017), and beef cattle (average 2.19) as found by (Simoni et al., 2021).

Total-tract apparent and true digestibility estimated through uNDF and AIA

Before discussing our results, it's important for the reader to consider that uNDF does not have a stable circadian excretion pattern, therefore its reliability depend on the sampling protocol used; apparently, this risk could be lower in the case of AIA (Thonney et al., 1985). Additionally, the diet analyzed was sampled at the pen level, but the faeces were individual, which may further exacerbate this digestibility bias. These considerations should be taken into account when interpreting digestibility data, also considering that field studies conducted on commercial farms often come with inherent limitations.

The descriptive statistics of total tract apparent digestibility (tta) and total tract digestibility (tt) using various internal markers are provided in Tables 3 and 4.

Considering ttaD and ttD estimate by means of uNDF, apart from ttaStarchD and ttaEED, which average values were higher than 846.7 g/kg, average ttaDMD, ttaOMD and ttaCPD were above 648.5 g/kg. The means of all the other parameters were lower than 500.7 g/kg. The variability ranged between 0.9% (ttaStarchD) and 74.4% (ttaADIND).

Considering ttaD and ttD estimate by means of AIA, except from ttaStarchD, ttaEED, and ttaOMDF which had average values higher than 720.4 g/kg, ttaCPD, ttaDMD and ttaCelD resulted above 602.1 g/kg. The means of all the other parameters were lower than 589.6 g/kg. The variability ranged from 0.9% (ttaStarchD) to 66% (ttADIND).

Table 3. Descriptive statistics of average estimated total-tract apparent (tta) and true (tt) nutrients digestibility (D) using fecal uNDF as a marker in feces of lactating buffaloes (g/kg).

Trait[1]	mean	SD[2]	min	max	CV[3]
ttaDMD	648.5	35.9	550.4	747.3	55.36
ttaOMD	675.9	33.84	577.1	773.9	50.07
ttaEED	846.7	85.7	603.3	951.5	101.22
ttaNDFomD	529.4	49.4	413.7	642.9	93.31
ttNDIND	385.3	177	42.86	695.9	459.38
ttADFomD	500.7	86.7	313.2	696.8	173.16
ttADIND	365.3	27.2	5.8	852.9	744.59
ttADLD	274.4	97.6	31.59	482	355.69
ttHemicelD	483.7	109.5	164	749.1	226.38
ttCelD	539	83.8	251.3	740.8	155.47
ttaCPD	655.2	52.7	491.5	759.5	84.3
ttaStarchD	973.3	9.54	947.9	995.1	9.80

[1]ttaDMD=total tract apparent dry matter digestibility; ttaOMD=total tract apparent organic matter digestibility; ttaEED=total tract apparent EE digestibility; ttNDFomD=total tract amylase treated neutral detergent fibre corrected by residual ashes; ttNDIND= total tract N bound to NDF digestibility; ttADFomD=total tract acid detergent fibre digestibility corrected by residual ashes; ttADIND= total tract N bound to ADF digestibility; ttADLD= total tract ADL digestibility; ttHemicelD=total tract hemicellulose digestibility; ttCelD=total tract cellulose digestibility; ttaCPD=total tract apparent crude protein digestibility; ttaStarchD= total tract Starch digestibility.
[2]SD=standard deviation.
[3]CV=coefficient of variation (%).

Table 4. Descriptive statistics of average estimated total-tract apparent (tta) and true (tt) nutrients digestibility (D) using fecal AIA as a marker in feces of lactating buffaloes (g/kg).

Trait[1]	mean	SD[2]	min	max	CV[3]
ttaDMD	695.7	41.5	573.5	792.9	59.65
ttaOMD	720.4	40.5	600.2	811.5	56.22
ttaEED	866.7	80.8	637.3	963.5	93.23
ttaNDFomD	589.6	73.3	413.1	743.7	124.32
ttNDIND	439.6	209	48.44	754.9	475.43
ttADFomD	563.1	102.3	309.5	748.1	181.67
ttADIND	290.9	192.1	4.57	638	660.36
ttADLD	364.6	120.5	3.91	771.9	330.50
ttHemicelD	541.6	133.1	197.1	800.8	245.75
ttCelD	602.1	72.7	380.2	760.6	120.74
ttaCPD	701.2	54	540.9	823.7	77.01
ttaStarchD	977.3	8.36	949.5	995.7	0.09

[1]ttaDMD=total tract apparent dry matter digestibility; ttaOMD=total tract apparent organic matter digestibility; ttaEED=total tract apparent EE digestibility; ttNDFomD=total tract amylase treated neutral detergent fibre corrected by residual ashes; ttNDIND= total tract N bound to NDF digestibility; ttADFomD=total tract acid detergent fibre digestibility corrected by residual ashes; ttADIND= total tract N bound to ADF digestibility; ttADLD= total tract ADL digestibility; ttHemicelD=total tract hemicellulose digestibility; ttCelD=total tract cellulose digestibility; ttaCPD=total tract apparent crude protein digestibility; ttaStarchD= total tract Starch digestibility.
[2]SD=standard deviation.
[3]CV=coefficient of variation (%).

The comparison of total tract and true digestibility of nutrients estimated through the use of uNDF and AIA is reported in Table 5.

The use of uNDF has shown to offer more accurate predictions of nutrient digestibility in lactating cows, when compared with AIA, considering total fecal collection as gold standard (C Lee and Hristov, 2013; Simoni et al., 2024). However, as observed by Morris et al. (2018), the accuracy of uNDF as a marker can be influenced by multiple factors, including diet typology, marker digestibility, analytical methods, sampling protocols, and the species of interest.

In a study conducted on lactating dairy cattle, a strong relationship (r = 0.93) was found between iNDF intake and fecal flow of iNDF, with an average recovery rate of 1.01 g/g across different forages, though this ranged widely depending on the type of forage. Notably, the highest iNDF recovery was observed in maize silage-based diets, which resulted in a possible underestimation of

nutrient digestibility when using other forages (Lund et al., 2007). Consistent with these findings, our study also found that digestibility estimates using uNDF were lower compared to those obtained using AIA. Similar trends were observed in buffaloes, where uNDF was shown to underestimate digestibility compared to total collection, likely due to variability in marker recovery (Maeda et al., 2011; Soares et al., 2011). Conversely, AIA demonstrated near-complete recovery (around 100%) in various species, including buffalo, which suggests that it may provide more reliable digestibility estimates, especially when sampling is conducted only once a day, which is common in many experimental setups (Sales and Janssens, 2003). Furthermore, AIA is unaffected by daily variation, making it a more stable marker under certain conditions (Thonney et al., 1985).

On a similar note, our results showed that the digestibility of fibrous components, such as ttNDFomD and ttADFomD, were statistically lower when using uNDF compared to AIA. This suggests that while uNDF might offer a more conservative estimate of fiber digestibility, it might also introduce underestimations in certain contexts. The inconsistency between uNDF and AIA across different variables (e.g., ttaDMDe, ttaOMDe, and ttADFDe) underscores the importance of considering the specific conditions under which each marker is used.

Table 5. Comparison of the descriptive statistics of average total-tract apparent (tta) and total-tract (tt) nutrients digestibility (D) estimated using faecal uNDF and AIA as internal marker.

	Estimated digestibilty with markers (%)			
Trait[1]	uNDF	AIA	SEM	p-Value
ttaDMD	64.85	69.57	5.55	<0.0001
ttaOMD	67.59	72.04	5.76	<0.0001
ttaEED	84.67	86.67	7.07	0.0003
ttNDFomD	52.94	58.96	4.62	<0.0001
ttNDIND	38.53	43.96	3.40	0.0164
ttADFomD	50.07	56.31	4.39	<0.0001
ttADIND	36.53	29.09	2.97	0.1495
ttADLD	27.44	36.46	2.64	<0.0001
ttHemicelD	48.37	54.16	4.23	0.0001
ttCelD	53.9	60.21	4.71	<0.0001
ttaCPD	65.52	70.12	5.60	<0.0001
ttaStarchD	97.33	97.73	8.05	0.0002

[1]ttaDMD=total tract apparent dry matter digestibility; ttaOMD=total tract apparent organic matter digestibility; ttaEED=total tract apparent EE digestibility; ttNDFomD=total tract amylase treated neutral detergent fibre corrected by residual ashes; ttNDIND= total tract N bound to NDF digestibility; ttADFomD=total tract acid detergent fibre digestibility corrected by residual ashes; ttADIND= total tract N bound to ADF digestibility; ttADLD= total tract ADL digestibility; ttHemicelD=total tract hemicellulose digestibility; ttCelD=total tract cellulose digestibility; ttaCPD=total tract apparent crude protein digestibility; ttaStarchD= total tract Starch digestibility.

In the following figures, namely 1, 2, and 3, we evaluated the graphical representations of the linear regressions between uNDF (y; %DM) and AIA (x; %DM), together with their Spearman's correlation coefficients, for ttaDMD, ttNDFD, and ttaCPD. The parameters indicated that, overall, when predicting ttaCPD, the relationship between uNDF and AIA, both evaluated through R^2 and r, is stronger than when estimating ttNDFD and ttaDMD. This is further confirmed by the slopes, which decrease numerically from ttaCPD to ttaDMD. Our results suggest that uNDF and AIA predict closer estimated of ttaDMD, compared to and followed by ttNDFD and ttaDMD, respectively.

Figure 1. Graphical representation of the linear regression between different markers (y; uNDF, %DM) plotted against AIA (x; % DM), for ttaDMD.

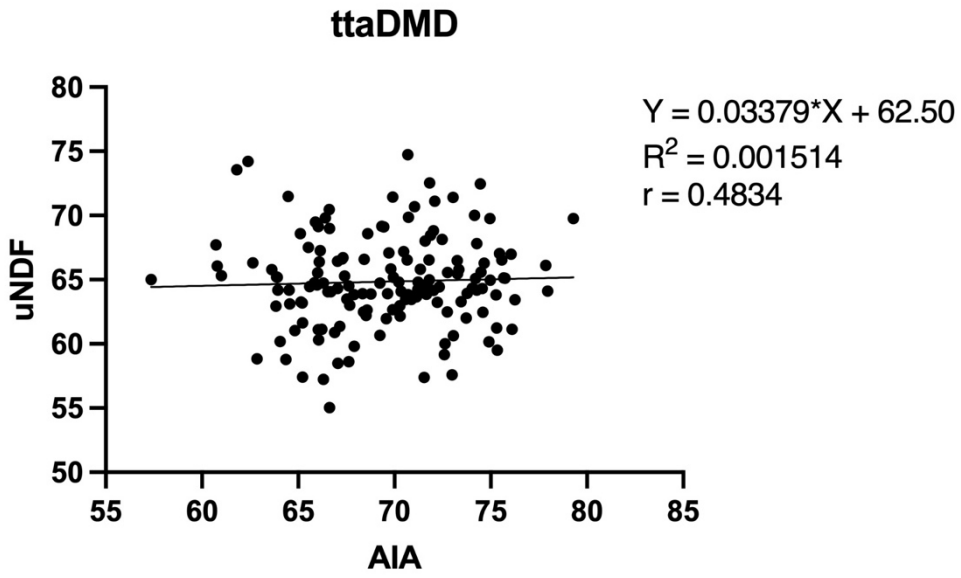


Figure 2. Graphical representation of the linear regression between different markers (y; uNDF, %DM) plotted against AIA (x; % DM), for ttNDFD.

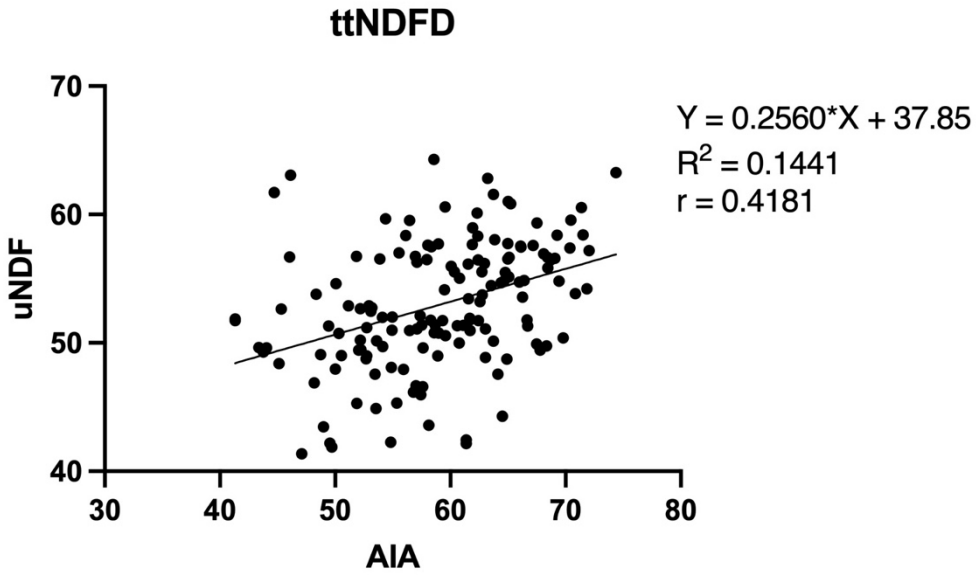
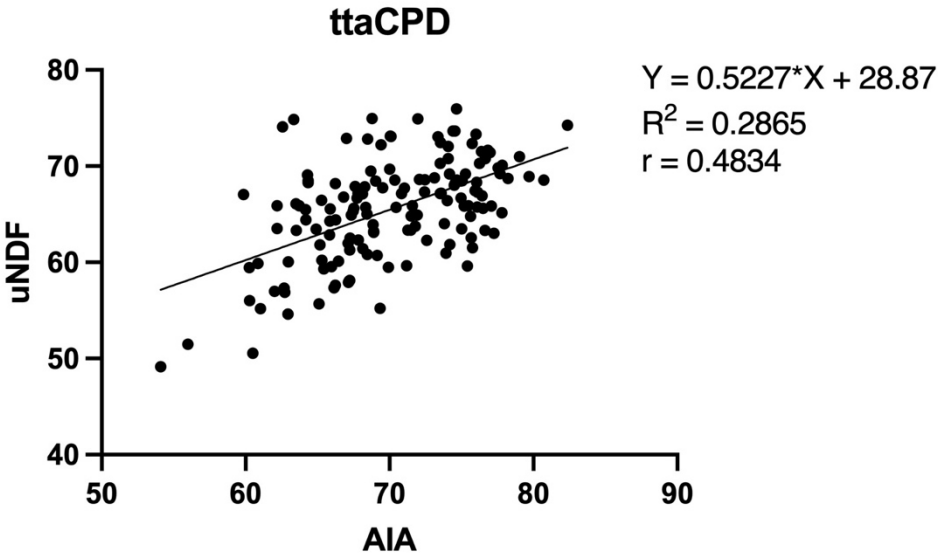


Figure 3. Graphical representation of the linear regression between different markers (y; uNDF, %DM) plotted against AIA (x; % DM), for ttaCPD.



Conclusions

The results of this study highlight important considerations when using uNDF and AIA as internal markers to estimate nutrient digestibility in lactating Mediterranean buffaloes, fed silage-based diets. Despite the ability of uNDF's to provide conservative estimates of fiber digestibility, it can present challenges due to its lack of a stable circadian excretion pattern, as reported in literature and briefly also by Trial 1.

Our analysis shows that, in most cases, AIA provided higher digestibility estimates than uNDF across various parameters, which may suggest that AIA could offer a more reliable picture of digestibility, especially in high-fiber diets, or conversely this difference in estimating fiber fractions, could be due to the tendency of ashes to inflate digestibility when using AIA compared to uNDFom. Lastly, our results suggest that, when considering ttaCPD, the two internal markers perform closer than when used to predict ttaDMD and ttNDFD.

In conclusion, while AIA may offer more reliable and stable estimates of nutrient digestibility in some cases, especially in commercial or field-based studies, uNDF provides conservative estimates

but is prone to underestimation in certain contexts. This topic will be further evaluated when considering dairy cows, namely in the third study.

Trial 3. Comparison of mean values of total tract apparent and true digestibility estimate through uNDF and uNDFom in dairy cows

Materials and Methods

Samples collection and chemical analysis

A total of 81 faecal samples (600 g) randomly selected across the lactating groups of 27 herds located in northern Italy were collected directly from the rectum of Holstein Friesian and crossbred dairy cows. Eight herds were sampled in the Parmigiano Reggiano region, a second set of eight farms were sampled in the central-east part of Veneto region while the remaining eleven farm from Piemonte region. Between the all farms, 33 were a corn-silage based total mixed ration (TMR), 30 were hay based TMR and 18 were using other silages; each farm was samples once a month, for 3 consecutive months. Dietary samples were collected over two days before faecal collection. Post TMR collection, the samples were stored at −20°C at different locations, and later analyzed at the University of Parma (Parma, Italy).

Both TMR and faecal samples were oven-dried at 55°C until they reached a constant weight, then ground using a Retch SK mill (Bauknecht, Stuttgart, Germany) to pass through a 1-mm screen. Dry matter (DM), ash, and ether extract (EE) were analyzed according to European Commission Regulation No. 152/2009. Amylase-treated neutral detergent fiber without residual ash (aNDFom), acid detergent fiber without residual ash (ADFom), and acid detergent lignin (ADL) were analyzed using heat-stable amylase and corrected for ash (Mertens et al., 2002). Nitrogen (N) were measured by combustion digestion at 900°C in excess oxygen using a Dumatherm (Gerhardt GmbH & Co, Königswinter, Germany) as described by Mihaljev et al. (2015). Starch content was determined enzymatically (method 2014.10; AOAC International, 2014). The undigested neutral detergent fiber (uNDF) content was determined through 240-hour fermentation (Raffrenato et al., 2018) in an in vitro batch system, using rumen fluid from four cows, as described by Simoni et al. (2020).

Digestibility Calculations and statistical analysis

The estimated apparent total-tract (tta) digestibility (D) of DM (ttaDMD), ash (ttaAsh), Crude Protein (ttaCPD; CP), Starch (ttaStarchD) and total-tract (tt) D of aNDFom (ttaNDFomD), ADFom

(ttADFomD), were calculated using both uNDF and uNDFom the of the TMR and of the related faecal samples as internal markers as described by Fustini et al. (2017).

Statistical analyses were performed using Prism. Normality was assessed graphically and by using the Shapiro–Wilk test. Since digestibility of nutrient were not normally distributed, a Mann-Whitney test was used to compare digestibility values for each nutrient between markers (uNDF vs uNDFom). Linear regressions models were applied between uNDFom (y; %DM) plotted against uNDF (x; %DM), to evaluate the relationship between the predictive abilities of the two markers, considering the estimation of ttaDMD, ttNDFD and ttaCPD. Furthermore, a Sperman's correlation coefficient was applied to evaluate the relationship between uNDFom and uNDF, used to estimate ttaDMD, ttNDFD and ttaCPD.

Results and Discussion

Dietary and faecal chemical composition

The analyzed dietary chemical composition, divided by the three main forage inclusion, are shown in Table 1.

The DM content was higher in the mix silage-based diet, followed by hay-based and corn silage-based diets. (919 vs 742 vs 699 g/kg). The ash content was higher in the hay-based diet compared to the other dietary forage-inlcusions, ranging from 72.7 to 87 g/kg DM. The CP content was consistent between forages inclusion, ranging from 162 to 150 g/kg, depending on forage inclusion. As expected, fiber fractions were higher in the hay-based diet, with aNDFom at 403 g/kg DM and ADFom at 237 g/kg DM; uNDF and uNDFom were also higher in the hay-based diet (154 and 169 g/kg DM, respectively). Starch content was higher in the corn silage-based diet (237 g/kg DM).

Table 1. Average chemical composition and standard deviation (±) of TMR, based on different king of forage inclusion.

Item[1]	Hay-based			Corn Silage-based			mix Silages-based		
Chemical composition, g/kg of DM unless otherwise indicated									
DM	742	±	139	699	±	204	919	±	11.3
Ash	87	±	22	72.7	±	7.56	81.2	±	9.56
CP	150	±	12.6	155	±	13.6	162	±	15.1
aNDFom	403	±	42.5	353	±	43.1	308	±	41.4
ADFom	237	±	20.9	207	±	29.8	183	±	37.2
Starch	166	±	54.3	237	±	60.3	221	±	44.8
uNDF	154	±	2.15	119	±	28.2	91.6	±	17.5
uNDFom	169	±	22.3	127	±	24.3	102	±	18.3

[1]DM= DM of pre-dried samples; aNDFom= amylase treated NDF without residual ash; ADF= ADFom without residual ash; uNDF= undigestible NDF evaluated after 240-h of fermentation and expressed as inclusive or residual ash; uNDFom= undigestible NDF evaluated after 240-h of fermentation and expressed as exclusive or residual ash.

The faecal composition, as report in Table 2, varied notably across diets, reflecting differences based on the dietary forage inclusion. While DM content was similar across all diets, ranging from 919 to 922 g/kg, fecal aNDFom was higher in the hay-based diet (578 g/kg DM), together with ADFom (365 g/kg DM). Fecal CP content averaged 167 g/kg DM, with the mix silages-based diet having an higher content (178 g/kg DM). Fecal starch was lower in the hay-based diet (11.8 g/kg DM) and higher in the corn silage-based diet (21.7 g/kg DM), as expected. The DM content of feces observed in our study (average 919 g/kg DM across all treatments) is consistent with values reported by (Righi et al., 2017), where the fecal DM content was reported as 92.8%; similar results were found by Mgbeahuruike et al. (2016) in cows fed grass-silage and corn-silage-based diets.

Similarly, the ash content of feces in our study (ranging from 127 to 148 g/kg DM) is comparable to the findings of an older study, where diets rich in fiber and minerals expectedly led to increased ash concentrations in the feces (Salo et al., 1970).

The aNDFom and ADFom ranges in our study's feces (520-578 and 331-365 g/kg DM, respectively) are consistent with those reported by Righi et al. (2017) where fecal NDF and ADF were 60.5% and 39.9% of DM, respectively, in cows fed hay-based diets. Similar trends were observed in both hay and silage-based diets in Brown lactating swiss cows (Hindrichsen et al., 2006) confirming that forages rich in indigestible fiber increase the fiber fractions in feces. On the contrary, when feeding dry cows with a hay-based diets, lower average values were reported (Kanani et al., 2014).

In our study, the starch content of feces ranged from 11.8 to 21.7 g/kg DM, from hay-based to corn-silage based diets. These values are lower than those reported by Bovera et al. (2007) in Mediterranean buffaloes but are comparable to the range found in lactating dairy cows by Righi et al. (2017), where fecal starch content was also low due to a probable efficient starch digestion.

The dietary uNDF content averaged 12.5 % DM, while fecal uNDF was higher at 33.5 % DM, resulting in a difference of +21 %. Similarly, dietary uNDFom was 13.2 % DM, compared to a fecal uNDFom content of 33 % DM, leading to a difference of +19.8 %.

The uNDF content of feces in our study (ranging from 322 g/kg DM to 355 g/kg DM) is within the range reported by Righi et al. (2017) and Cavallini et al. (2023), where uNDF in feces was found to be 40.4 and 34.8 % of DM, respectively.

Table 2. Chemical composition (g/kg DM) of dairy cows pre-exsiccated faeces, divided by dietary treatment.

Item[1]	Hay-based			CornSilage-based			Other Silages-based		
Chemical composition, g/kg of DM unless otherwise indicated									
DM (g/kg)	922	±	147	919	±	103	919	±	75
Ash	129	±	18.5	127	±	19.4	148	±	26.9
CP	154	±	6.9	160	±	14.7	178	±	14.4
aNDFom	578	±	2.48	564	±	32.3	520	±	40.4
ADFom	365	±	20.6	337	±	42.3	331	±	52.5
Starch	11.8	±	7.39	21.7	±	10.5	15.2	±	12.8
uNDF	355	±	35.5	328	±	44.2	322	±	51.8
uNDFom	357	±	37.5	327	±	45.1	308	±	35.1

[1]DM= DM of pre-dried samples; aNDFom= amylase treated NDF without residual ash; ADF= ADFom without residual ash; uNDF= undigestible NDF evaluated after 240-h of fermentation and expressed as inclusive or residual ash; uNDFom= undigestible NDF evaluated after 240-h of fermentation and expressed as exclusive or residual ash.

Descriptive statistics of the ttaD and ttD using uNDF and uNDFom as internal marker are presented in Table 3 and 4. Considering uNDF, apart from ttaStarchD, which average values was 973 g/kg, average ttaDMD and ttaCPD were belowe 586 g/kg. The means of all the other parameters were lower than 345 g/kg. The variability ranged between 1.8 % (ttaStarchD) and 47.5 % (ttaADFomD). Considering uNDFom, a similar numerical trend can be reported for ttaStarchD, which average values was 972 g/kg. The average values of all the other parameters were below 626 g/kg. The variability ranged between 1.7 % (ttaStarchD) and 40.4 % (ttaADFomD).

Table 3. Descriptive statistics of average total-tract apparent (tta) and total-tract true (tt) of nutrients digestibility estimated (D) using faecal uNDF as internal marker (g/kg).

Trait[1]	Mean	SD[2]	Minimum	Maximum	CV[3]
ttaDMD	586	91.2	361	759	155
ttNDFomD	345	129	28.3	609	376
ttADFomD	300	142	4.18	723	475
ttaCPD	583	91.5	323	761	155
ttaStarchD	973	17.7	912	999	18.2
ttaAshD	333	135	55.5	611	407

[1]ttaDMD=total tract apparent dry matter digestibility; ttNDFD=total tract amylase treated neutral detergent fibre, exclusive of residual ash; ttADFomD=total tract acid detergent fibre digestibility, exclusive of resisual ash; ttaCPD=total tract apparent crude protein digestibility; ttaStarchD=total tract apparent Starch digestibility; ttaAshD=total tract apparent ash digestibility.
[2]SD=standard deviation.
[3]CV=coefficient of variation.

Table 4. Descriptive statistics of average total-tract apparent (tta) and total-tract true (tt) of nutrients digestibility estimated (D) using faecal uNDFom as internal marker.

Trait[1]	Mean	SD[2]	Minimum	Maximum	CV[3]
ttaDMD	626	85.9	428	781	137
ttNDFomD	408	122	156	696	300
ttADFomD	370	149	99.8	882	404
ttaCPD	625	87.3	398	775	139
ttaStarchD	972	17.4	916	999	17.8
ttaAshD	397	125	133	647	316

[1]ttaDMD=total tract apparent dry matter digestibility; ttNDFD=total tract amylase treated neutral detergent fibre, exclusive of residual ash; ttADFomD=total tract acid detergent fibre digestibility, exclusive of resisual ash; ttaCPD=total tract apparent crude protein digestibility; ttaStarchD=total tract apparent Starch digestibility; ttaAshD=total tract apparent ash digestibility.
[2]SD=standard deviation.
[3]CV=coefficient of variation.

Total-tract apparent and true digestibility estimated through uNDF and uNDFom

The comparison of total tract and true digestibility of nutrients estimated using uNDF and uNDFom are reported in Table 5.

Between the two, uNDF consistently provides statistically higher estimates of ttaDMD compared to uNDFom. This difference can be attributed to the inclusion of residual ash in uNDF, which could inflate ttaD and ttD by overestimating the indigestible fraction. In high-ash diets, such as those with hay-based forages, this overestimation can hinder precise feed evaluation and efficiency optimization in dairy cows (Raffrenato et al., 2019).

Our study results reports that ttNDFomD is higher when estimated using uNDFom (408.6 vs 345 g/kg; p=0.0042). The exclusion of ash in uNDFom leads to a more accurate reflection of fiber digestibility, which is particularly crucial when considering ttD of fibrous fractions in ruminants. This makes uNDFom a better marker for fiber-rich diets, without difference of forage inclusion, as already widely recognized in literature (Sirois, 2015).

On the contrary, considering ttADFomD, uNDF provides significantly higher compared to uNDFom (370.5 vs 300.7 g/kg; p = 0.0036). ADF, representing a lesser digestible fiber fraction than NDF, is often overestimated by uNDF due to the inclusion of ash. By excluding ash, uNDFom provides a more conservative measure of digestibility, which is essential for evaluating feed quality and optimizing nutrient absorption, especially in high-silage diets (Raffrenato and Van Amburgh, 2010).

Considering ttaCPD, uNDFom yield a higher statistical ttaD compared to uNDF (625.1 vs 583.5 g/kg). There is no significant difference between uNDF and uNDFom when estimating starch digestibility (~973 g/kg). However, for ash digestibility, uNDF provides and expected inflated estimates compared to uNDFom (391.3 vs 333.8 g/kg). As outlined before, this difference highlights uNDF's tendency to overestimate digestibility in ash-heavy diets, such as those involving high-mineral forages: the residual minerals contaminate the NDF residue leading to an

overestimation of the fiber value, which may lead to rations that appear to be adequate in fiber, which in fact are deficient (Raffrenato et al., 2018).

Table 5. Comparison of ttaD and ttD of DM, NDFom, ADFom, CP, Starch and Ash estimated using uNDF and uNDFom, expressed as g/kg.

	Estimated digestibilty with markers (g/kg)			
Trait[1]	uNDF	uNDFom	SEM	p-Value
ttaDMD	626.6	586.2	10.097	0.0091
ttNDFomD	345.0	408.6	14.585	0.0042
ttADFomD	370.5	300.7	17.015	0.0036
ttaCPD	583.5	625.1	10.300	0.0061
ttaStarchD	972.7	974	2.462	0.6697
ttaAshD	391.3	333.8	15.720	0.0074

[1]ttaDMD=total tract apparent dry matter digestibility; ttNDFomD=total tract amylase treated neutral detergent fibre, exclusive of residual ash; ttADFomD=total tract acid detergent fibre digestibility, exclusive of residual ash; ttaCPD=total tract apparent crude protein digestibility; ttaStarchD=total tract apparent Starch digestibility; ttaAshD=total tract apparent ash digestibility.

Descriptive statistics of the ttaD and ttD estimated using uNDF and uNDFom, across the different inclusion of hay or silages in the diets, is reported in Table 6 and 7, respectively.

Hay-based diets, which typically contain more fibrous and lignified material compared to silage-based diets, are more likely to have a higher ash content, particularly from low-quality forage or excessively cut-to-the ground hays, which inflates uNDF estimates of ttaDMD (Sirois, 2015). For example, in our condition estimated ttADFomD using uNDF is higher than when using uNDFom (349 vs 278 g/kg, respectively), which more accurately excludes the ash component.

Corn silage-based diets, which is generally lower in fiber and ash compared to hay-based diets, tends to have a more consistent and digestible fiber profile. In these diets, both uNDF and uNDFom can perform well, but uNDFom might still offer a slight advantage in accuracy for predicting fiber digestibility (Lopes et al., 2015).

However, the overestimation by uNDF in corn silage-based diets is generally less pronounced, do to their potentially lower ashes content: to the author opinion, uNDF might still be acceptable if slight inaccuracies in fiber digestibility are admissible (Palmonari et al., 2016)

Diets based on mix-based silages, such as grass or legume silages, often provide higher fiber digestibility than hay-based diets but can vary more than corn silage in terms of ash content. In these cases, uNDFom can be preferred marker because it adjusts for ash content variability and provides more precise estimates for fiber fractions. Silages that have undergone fermentation may contain higher levels of ash due to mineralization processes during ensiling (Hansen and Spears, 2009), and uNDFom helps eliminate this bias, offering a more accurate reflection of how much fiber is truly digestible. Therefore, in silage-heavy diets where precision is crucial, especially in research or detailed feed optimization, uNDFom should be used to avoid overestimating fiber digestibility (Raffrenato and Van Amburgh, 2010; Sirois, 2015).

Table 6. Descriptive statistics (mean ± standard deviation) of ttaD and ttD of DM, NDFom, ADFom, CP, Starch and Ash estimated using uNDF, by different dietary forage inclusions, expressed as g/kg.

Item	Hay-based			CornSilage-based			Other Silages-based		
ttaDMD	588	±	86.5	635	±	80.3	697	±	80.2
ttNDFomD	371	±	119	411	±	108	512	±	142
ttADFomD	349	±	166	376	±	151	452	±	155
ttaCPD	582	±	84.5	634	±	88.3	684	±	82.4
ttaStarchD	970	±	19.6	969	±	20.1	978	±	14.2
ttaAshD	363	±	127	414	±	125	429	±	149

[1]ttaDMD=total tract apparent dry matter digestibility; ttNDFomD=total tract amylase treated neutral detergent fibre, exclusive of residual ash; ttADFomD=total tract acid detergent fibre digestibility, exclusive of residual ash; ttaCPD=total tract apparent crude protein digestibility; ttaStarchD=total tract apparent Starch digestibility; ttaAshD=total tract apparent ash digestibility.

Table 7. Descriptive statistics (mean ± standard deviation) of ttaD and ttD of DM, NDFom, ADFom, CP, Starch and Ash estimated using uNDFom, by different dietary forage inclusions.

Item	Hay-based			CornSilage-based			Other Silages-based		
ttaDMD	545	±	104	594	±	79.1	645	±	88.3
ttNDFomD	305	±	139	347	±	111	446	±	151
ttADFomD	278	±	181	297	±	115	408	±	140
ttaCPD	540	±	99.4	596	±	88.5	634	±	84.1
ttaStarchD	974	±	17.4	970	±	22.4	975	±	17.4
ttaAshD	301	±	145	351	±	132	361	±	167

[1]ttaDMD=total tract apparent dry matter digestibility; ttNDFomD=total tract amylase treated neutral detergent fibre, exclusive of residual ash; ttADFomD=total tract acid detergent fibre digestibility, exclusive of residual ash; ttaCPD=total tract apparent crude protein digestibility; ttaStarchD=total tract apparent Starch digestibility; ttaAshD=total tract apparent ash digestibility.

In the following figures, namely 1, 2, and 3, we evaluated the graphical representations of the linear regressions between uNDFom (y; %DM) and uNDF (x; %DM), together with their Spearman's correlation coefficients, for ttaDMD, ttNDFD, and ttaCPD. The parameters indicated that, overall, these two markers performed with a very similar level of accuracy, which decreased from ttaDMD to ttaCPD and ttNDFD.

Figure 1. Graphical representation of the linear regression between uNDFom (y; %DM) plotted against uNDF (x; % DM), for ttaDMD.

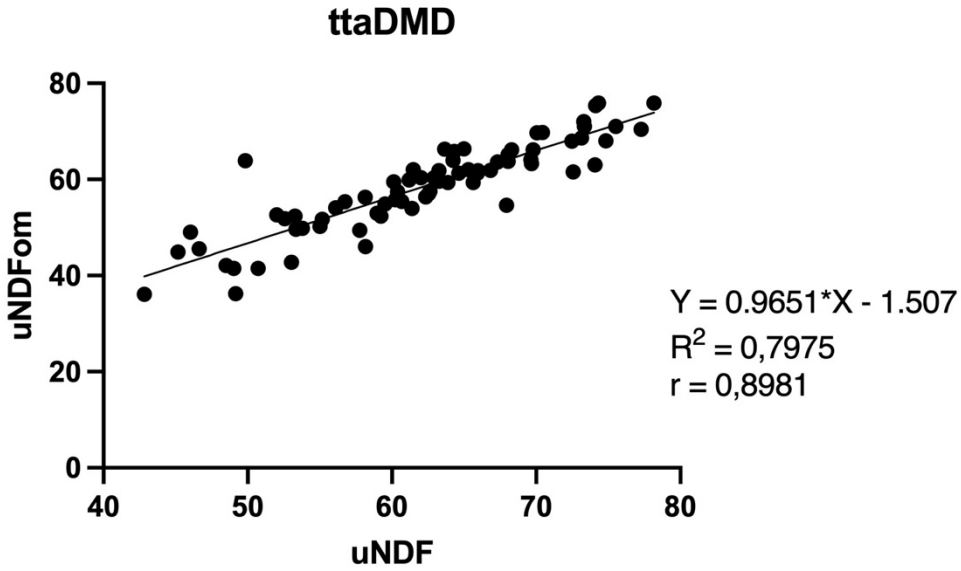


Figure 2. Graphical representation of the linear regression between uNDFom (y; %DM) plotted against uNDF (x; % DM), for ttNDFD.

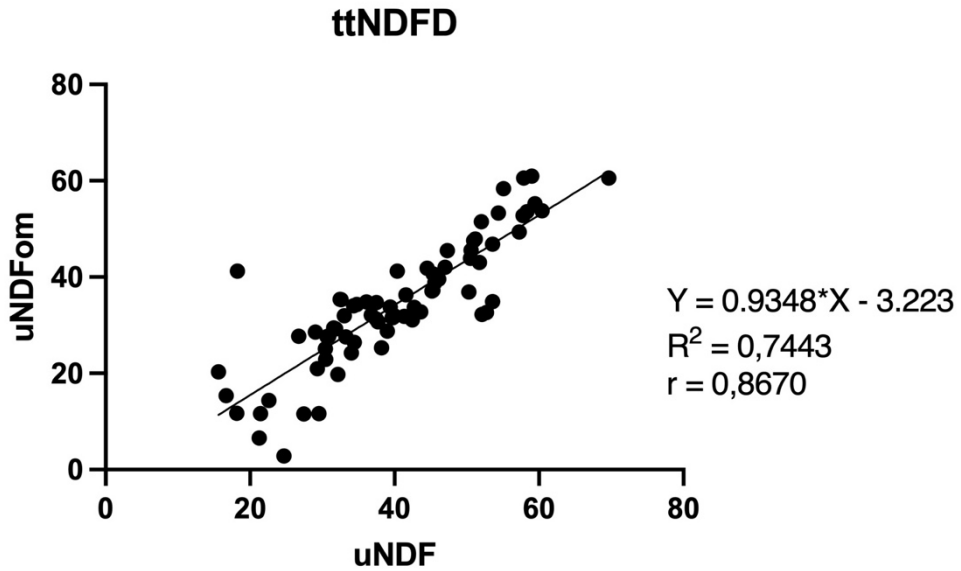
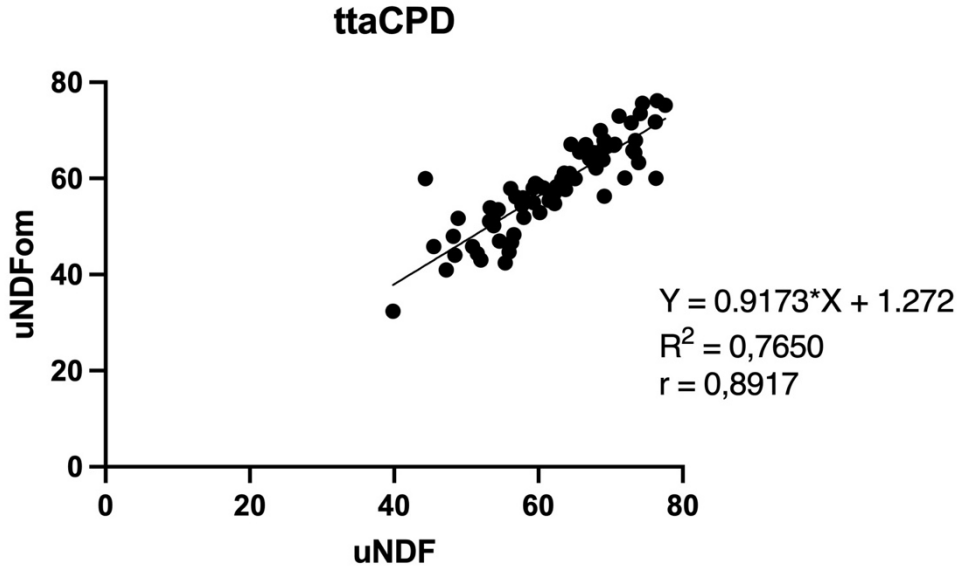


Figure 3. Graphical representation of the linear regression between uNDFom (y; %DM) plotted against uNDF (x; % DM), for ttaCPD.



Conclusions

Overall, this datas do not add any novelty to the already well-established concept that uNDFom is the preferred marker in forage-based diets, especially when the goal is to achieve precise estimates of fiber digestibility, as it focuses solely on organic matter without the interference of ash.

It is true that, considering dairy cows fed forage-based diets, across different forage type and inclusions, uNDF and uNDFom generally followed similar trends, confirmed also by the linear regressions and correlation coefficients between the two when estimating ttaDMD, ttNDFD and ttaCPD. However, uNDF continued to provide higher digestibility estimates for dry matter, while uNDFom often predicted higher NDF and ADF digestibility.

This makes uNDFom a better marker for fiber-rich diets, providing more consistent results regardless of forage inclusion.

Trial 4: Comparison of mean values of total tract apparent and true digestibility estimated through uNDF, ADL and ADIA in Beef cattle

Adapted from Simoni et al. (2021).

Materials and methods

Sample collection and chemical analysis

A total of 172 pools of faecal samples were randomly selected across growing groups on five farms located in the Veneto region (Northern Italy) over the course of one year, from a total of 1206 Charoilaise beef cattle. Additionally, 164 corresponding dietary samples were collected, with the number of collected samples being 32 from three farms, and 28 and 48 from the remaining two farms. Faecal samples were collected and pooled 24 hours post-feeding at 15 and 30 days after the cattle's arrival at each farm. Samples were taken from the clean floor from male (359.3 ± 49.07 kg initial body weight) and female (352.2 ± 47.37 kg initial body weight) young Charolaise cattle to obtain 5 kg total sample. Dietary samples, including a total mixed ration (TMR) whose ingredients and chemical composition were predicted by a ration formulation software, were collected from the feed bunk during TMR delivery to obtain a 2 kg aliquot.

Both TMR and faecal samples were oven-dried at 55°C for 72 hours and then ground in a Cyclotec mill (Tecator, Herndon, VA) to pass through a 1-mm screen. For both TMR and faecal samples, dry matter (DM) content was measured by drying the samples overnight at 103°C. The undigestible neutral detergent fiber (uNDF) was determined through a 240-hour in vitro fermentation, using the procedure described by Righi et al. (2017). Rumen fluid was collected at the slaughterhouse from four cows and processed according to Simoni et al. (2020). The neutral detergent fiber (aNDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were analyzed based on Van Soest et al. (1991). Heat-stable amylase was used for aNDF determination without sodium sulfite, and the values were expressed inclusive of residual ash. Acid detergent insoluble ashes were also calculated as the ashes of the ADL fraction (ADIA). The aNDF and ADF residues were analyzed for bound N determination (NDIN and NADIN). Nitrogen (N) content was determined by combustion digestion of the sample at 900°C in excess oxygen using Dumatherm® (Gerhardt GmbH & Co, Königswinter, Germany) based on Mihaljev et al. (2015).

Digestibility Calculations and statistical analysis

The estimated total-tract apparent (tta) and total tract (tt) digestibility (D) values for dry matter (ttaDMD), NDF (ttNDFD), ADF (ttADFD), crude protein (ttaCPD; CP), N bound NDF (ttNDIND), N bound ADF (ttAIND), and ash (ttaAshD) were calculated using uNDF, while ttaDMD, ttaNDFD and ttaCPD were calculated also using ADL and ADIA, from TMR samples and from the corresponding faecal samples as described by Righi et al. (2017) and Bergero et al. (2004). Statistical analyses were performed using Prism. Normality was assessed using the Shapiro–Wilk test. Since digestibility of nutrient were not normally distributed, a Dunn's multiple comparison test was used to compare digestibility values for each nutrient between marker-estimated digestibility (uNDF vs ADL vs ADIA). Linear regressions models were applied between uNDF amd ADL (y; %DM) plotted against the ADIA (x; %DM), to evaluate differences in predictive ability of the markers employed, considering the estimation of ttaDMD, ttNDFD and ttaCPD. Furthermore, a Spearman's correlation coefficient was utilized to evaluate the relationship between ADIA and the before mentioned markers, used to estimate ttaDMD, ttNDFD and ttaCPD.

Results and Discussion

Dietary and faecal chemical composition

Dietary ingredients of the TMR fed to the animals and its calculated chemical composition is reported in Table 1.

The feedstuffs provided in the study included three types of supplements, employed in the different growing stages of the animals.

The dietary chemical composition was estimated using the ration formulation software NDS Professional version 3.9.9.05 (RUM&N Sas, Reggio Emilia, Italy).

The composition of the faeces, as presented in Table 3, shows considerable variability, likely due to differences in TMRt. The coefficient of variation (cv) for faecal components was over 12%, except for DM, aNDF, and N, which had cv values of 2.4%, 6.9%, and 8.7%, respectively. The faecal NDF content was 56.4% of DM, with 62.2% of that being ADF, and 27.2% lignin. Faecal N content was 2.6% of DM, with 27.4% bound to the NDF fraction and 26.9% bound to the ADF fraction.

The ash content in faeces was 62% higher than the ash content in the diet.

Overall, fecal composition measured in this study align within the ranges reported in the literature for beef cattle, indicating consistency with existing research. the coefficients of variation (cv) for most faecal components exceeded 12%, with the exceptions being DM, aNDF, and N, which showed cvs of 2.4%, 6.9%, and 8.7%, respectively. This variability can be attributed to the diverse diet and feeding practices common in beef production systems, where cattle consume a mix of roughages and concentrates, leading to fluctuations in the nutritional composition of their feed. A more comprehensive analysis of the faecal chemical composition, in the context of specific dietary conditions, and for an in-depth discussion on this topic please refer to Simoni et al. (2021).

Table 1. Dietary ingredients (kg as fed) of the TMR fed to the animals and its calculated chemical composition (g/kg).

Item	mean	sd	min	max	cv
Diet ingredient, kg as fed					
Feed stuff[1]	6.60	2.70	4.00	10.0	0.40
Wheat middlings	10.0	0.00	10.0	10.0	0.00
Distiller	7.50	2.10	5.00	10.0	0.28
Corn gluten meal	5.00	2.80	3.00	7.00	0.56
Alfalfa hay, 15% CP	2.00	0.00	2.00	2.00	0.00
Grass hay	8.00	2.80	6.00	10.0	0.35
Corn meal	13.3	5.10	9.00	19.0	0.38
Cane molasses	4.20	0.80	3.50	5.00	0.18
Wheat straw	14.0	2.70	10.0	16.0	0.19
Earlage	13.8	8.80	7.50	20.0	0.64
Dry Beet Pulp	5.30	4.60	0.00	10.0	0.87
Corn Silage, 36 % DM	47.5	17.1	30.0	70.0	0.35
Soybean meal, 46 %CP	3.00	0.00	3.00	3.00	0.00
Soybean hulls	3.50	0.00	3.50	3.50	0.00
Estimated diet composition, % DM					
DM, % as fed	624.1	41.2	568.9	667.4	0.60
CP	126.2	1.6	124.3	128.3	0.13
EE	33.1	1.20	31.9	34.7	0.36
CF	187.1	11.4	172.4	200.2	0.61
Ash	64.9	16.8	41.2	78.4	0.26
NDF	412.0	24.3	392.5	443.4	0.59
Starch	239.5	19.3	210.8	253.2	0.81

[1]Feed stuff: Bull one, bull 100 or bull 1500. Ingredients described in the text.
[2]Earlage: DM 64%, Starch 58% and NDF 14%.

The actual dietary samples' average chemical composition is reported in Table 2.

Table 2. Descriptive statistics of the average chemical composition of the diets provided to the animals, as % of DM.

Trait[1]	mean	sd[2]	min	max	cv[3]
DM	949	10.6	917	970	11.1
aNDF	401	54.6	228	617	136
NDIN	8.50	1.70	5.50	13.3	198
ADF	217	27.9	127	275	128
ADIN	72.0	1.50	4.50	13.1	213
ADL	406	11.9	18.3	135	294
ADIA	715	16.3	27.0	141	227
N	205	1.70	16.4	26.2	83.3
Ash	719	16.6	27.0	141	231
uNDF	105	28.1	30.6	199	265

[1]DM= DM of pre-dried samples; aNDF= amylase treated NDF with residual ash; NDICP= protein bound to NDF; ADF= ADF with residual ash; ADICP= protein bound to ADF; ADL=ADL expressed as exclusive of residual ash; ADIA= acid detergent insoluble ahses; uNDFom= undigestible NDF evaluated after 240-h of fermentation and expressed as exclusive or residual ash.
[2]sd= standard deviation.
[3]cv= variation coefficient.

Table 3. Average chemical composition of the feaces of the animals, as g/kg.

Trait[1]	mean	sd[2]	min	max	cv[3]
DM	937	22.4	793	997	23.9
aNDF	564	39.0	445	641	69.2
NDIN	7.20	1.10	4.00	10.3	150
ADF	351	48.3	223	557	137
ADIN	71.0	1.80	4.70	18.8	249
ADL	153	60.4	56.3	396	393
ADIA	116	13.6	84.3	156	117
N	26.3	2.30	15.0	32.4	87.2
Ash	116	13.4	84.3	156	114
uNDF	337	49.1	209	525	145

[1]DM= DM of pre-dried samples; aNDF= amylase treated NDF with residual ash; NDICP= protein bound to NDF; ADF= ADF with residual ash; ADICP= protein bound to ADF; ADL=ADL expressed as exclusive of residual ash; ADIA= acid detergent insoluble ahses; uNDFom= undigestible NDF evaluated after 240-h of fermentation and expressed as exclusive or residual ash.
[2]sd= standard deviation.
[3]cv= variation coefficient.

Total-tract apparent and true nutrients digestibility estimated throught uNDF, ADIA and ADL

Descriptive statistics of the ttaD and ttD using uNDF and ttaDMD, ttNDFD and ttaCPD using ADIA and ADL, as internal markers, are presented in Table 4. Considering uNDF, the average ttaDMD, ttNDFD and ttaCPD were above 610 g/kg. The means of all the other parameters were lower than 518.6 g/kg. The variability ranged between 13.5 % (ttaDMD) and 35.3% (ttaAshD).

Considering ttaDMD estimate with the use of ADL and ADIA, they differed by + 2.40 and - 29.13 % compared to ttaDMD estimated with uNDF, respectively.

Table 4. Average total-tract apparent (tta) and total tract (tt) nutrients digestibility (D) using dietary and faecal uNDF, ADIA and ADL as an internal marker (g/kg).

Trait[1]	mean	sd[2]	min	max	cv[3]
by means of uNDF					
ttaDMD	682.3	92.5	329.5	874.7	135.6
ttNDFD	679.2	121.7	72.6	971.2	179.1
ttNDIND	518.6	162.1	116.3	859.9	312.5
ttADFD	512.0	103.4	215.1	777.3	201.9
ttADIND	468.1	165.3	128.6	871.2	353.2
ttaCPD	601.8	110.5	214.2	845.3	183.7
by means of ADIA					
ttaDMD	391.0	126.9	2.40	781.3	324.6
ttNDFD	223.3	115.6	6.691	691.9	517.9
ttaCPD	609.9	119.2	152.9	831.1	196.5
by means of ADL					
ttaDMD	706.2	123.8	273.2	913.8	175.4
ttNDFD	590.2	164.8	26.73	889.3	279.2
ttaCPD	437.4	206.3	8.994	810.7	471.7

[1]ttaDMD=total tract apparent dry matter digestibility; ttNDFD=total tract amylase treated neutral detergent fibre; ttNDIN= total tract nitrogen bound to NDF digestibility; ttADFD=total tract acid detergent fibre digestibility; ttADIN=total tract nitrogen bound to ADF digestibilty; ttaCPD=total tract apparent crude protein digestibility.
[2]sd= standard deviation.
[3]cv= variation coefficient.

The descriptive statistics and comparison of ttaDMD estimated using the different markers, namely uNDF, ADL, and ADIA are presented in Table 5.

The results indicate significant differences among the markers, with ttaDMD being the highest when estimated using ADL (70.89%), followed by numerically lower, but not statistically different uNDF (68.89%), and lowest when using ADIA (41.28%). This trend is aligned with previous research, such as Huhtanen et al. (1994), which highlighted the variability of different markers, showing that ADL and uNDF generally yield more reliable estimates than ADIA.

Cochran et al. (1986) emphasized that marker selection is critical when predicting *in vivo* digestibility, noting that ADL and other acid detergent-based markers often produce inconsistent results due to incomplete recovery and variability in their behavior during gastrointestinal transit. This study further supports that ADL, despite providing higher digestibility estimates, comes with greater variability, making it less reliable for precise measurements. On a similar note, Velásquez et al. (2021) emphasized that the choice of marker not only impacts digestibility estimates but also has broader implications for assessing feed efficiency, which is of particular interest in beef cattle.

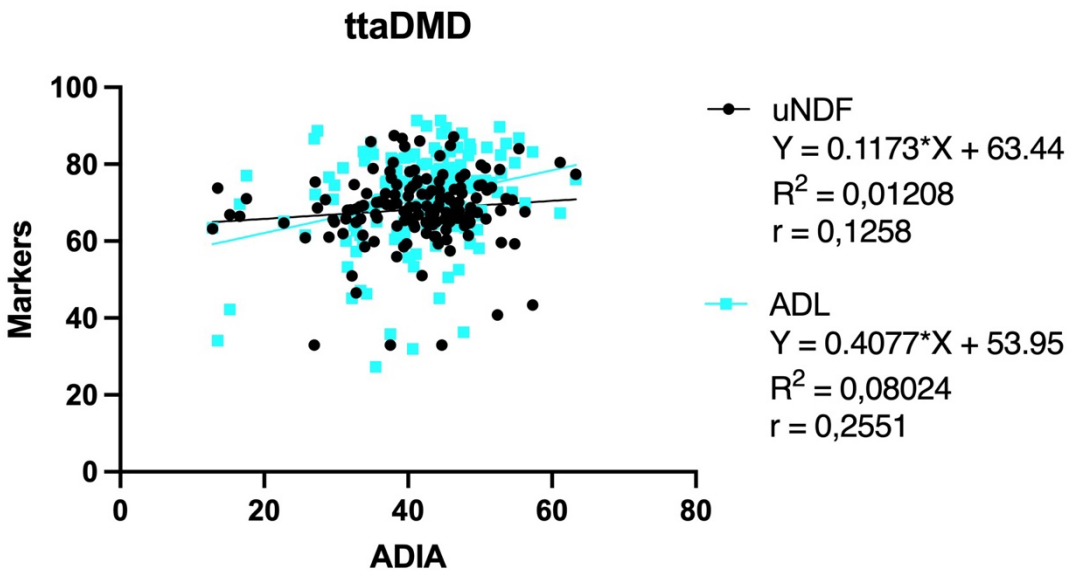
Table 5. Descriptive statistic and comparison of ttaDMD estimated using uNDF, ADL and ADIA (g/kg).

	uNDF		ADL		ADIA	
mean	688.9	a[1]	708.9	a	412.8	b
sd[2]	79.4		119.3		88.0	
min	408.3		319.5		128.1	
max	874.7		913.8		633.0	
cv[3]	110		160		210	

[1]*P* value <0,0001; Sem 0.76
[2]sd= standard deviation
[3]cv=variation coefficient

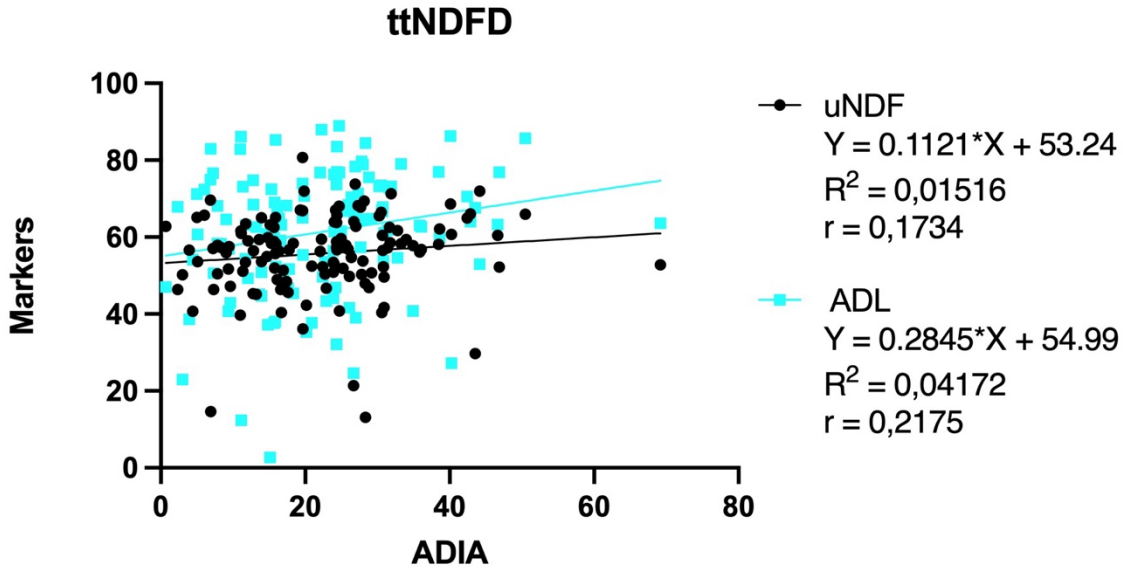
In the following Figures, namely 1,2 and 3, we evaluated the graphical representations of the linear regressions between uNDF and. ADL (y; %DM) and AIA (x; %DM), togheter with their Sperman's correlation coefficients, for ttaDMD, ttNDFD and ttaCPD.

Figure 1. Graphical representation of the linear regression between uNDF or ADL (y; %DM) plotted against ADIA (x; % DM), for ttaDMD.



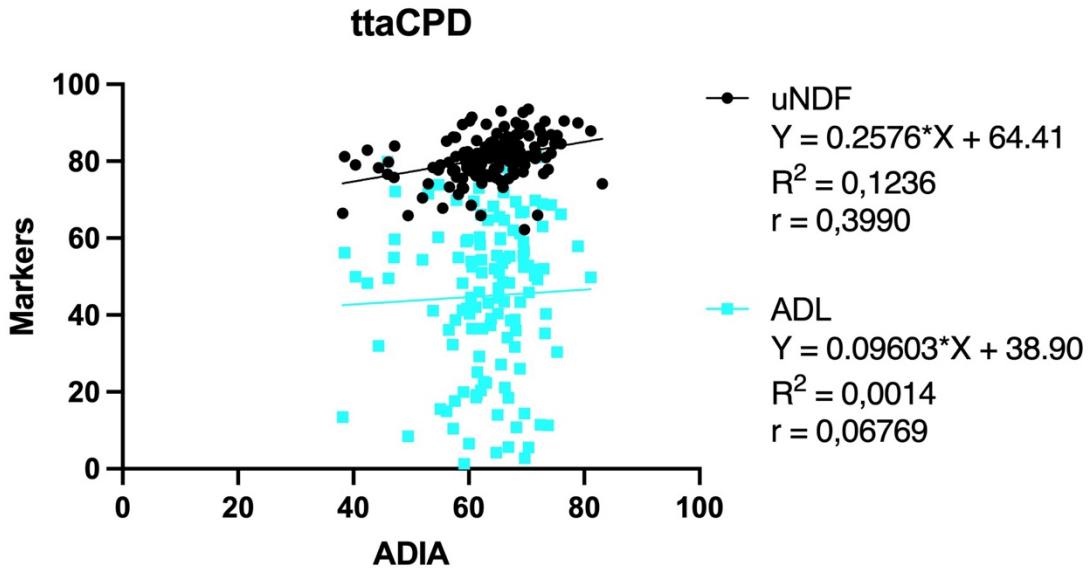
In Figure 1, ADL proved to estimate ttaDMD with a closer accuracy than uNDF, compare the ADIA, which can be considered as the gold standard when the total collection is not available (Lee and Hristov, 2013); this was proved by the higher R^2 (0.08 vs 0.01), r (0.25 vs 0.12) and slope (0.40 vs 0.11).

Figure 2. Graphical representation of the linear regression between uNDF or ADL (y; %DM) plotted against ADIA (x; % DM), for ttNDFD.



Similarly, as shown in Figure 2, ADL proved to estimate ttNDFD with a closer accuracy to AIA, than uNDF; on the contrary, when estimating ttaCPD, uNDF provided more reliable results than ADL, compared to ADIA.

Figure 3. Graphical representation of the linear regression between uNDF and ADL (y; %DM) plotted against ADIA (x; % DM), for ttaCPD.



Conclusions

This study, conducted on beef cattle fed silage-based diets, reinforces the already well-established understanding that uNDF and ADL are commonly preferred markers for measuring nutrient digestibility in beef cattle but adds nuance to the discussion by demonstrating how their performance compares to ADIA. In particular, the results confirm that uNDF and ADL provide higher ttaDMD estimates than ADIA, with ADL giving the highest values. However, despite its superior estimates, ADL exhibited greater variability, making uNDF a more consistent choice for digestibility assessments, specifically when considering ttaCPD.

Ethical approvals

Trial 1: Animal handling, housing, and care followed the Ethical Committee guidelines of the Department of Animal Science of the Agricultural University of Athens (EU 63/2010; Council of the European Union 2010).

Trial 2, 3, 4 and 5: According to Italian law on animal experimentation and ethics (DL 04/03/2014 n. 26), this study does not require ethical approval. The trial has been conducted in a responsible manner without affecting animal health and behavior.

Trial 5: Technical implications in the use of fecal markers for Nitrogen balance assessment of dairy cows fed hay-based diets in the Parmigiano Reggiano region.

Adapted from Danese et al. (2023), presented as oral presentation at the European Federation of Animal Science, 73rd annual meeting.

Introduction

Nitrogen (N) management in dairy farming hinges on two primary metrics: N use efficiency (NUE) and N balance (NB). While NUE provides insights into how efficiently cows utilize ingested N for productive outputs like milk, NB quantifies the difference between N intake and N excretion. Both metrics are essential for evaluating the sustainability and environmental impact of dairy operations.

The calculation of NUE is done as the ratio of N retained in milk to the total N intake. Low-moderate NUE values, typically around 25%, indicate that most of the dietary N is excreted and not efficiently utilized, primarily through feces and urine (Calsamiglia et al., 2010). This not only represents an inefficiency in resource use but also contributes to environmental pollution, as excreted N can volatilize as ammonia or leach as nitrate, leading to greenhouse gas emissions and water contamination. Improving NUE involves adjusting diets to better match cows' requirements, thereby reducing N waste. Research has shown that precision feeding strategies—such as balancing for essential amino acids and optimizing energy-to-protein ratios—can enhance NUE.

For example, Lapierre (2021) reported that optimizing diets for glucogenic nutrients and essential amino acids improved NUE to 34.3%, reducing the environmental footprint of N losses.

In addition to NUE, NB offers a comprehensive view of N dynamics by comparing N intake with excretions through milk, urine, and feces: NB is essential for understanding the net N impact of dairy farming, as it accounts for total N flows within the system.

This parameter is calculated and the difference between intake and excretion: the intake is considered as the daily total N intake, while the excretion is the sum of daily total fecal N output, daily urinary N output and daily milk N.

A negative NB indicates that cows are excreting more N than they are ingesting, which can signify inefficiencies in dietary N use or an incapability of digestion of the N fed to the animals. On the other hand, a positive NB implies N retention, which, while beneficial for milk production, can exacerbate N losses through increased excretions (Spanghero and Kowalski, 2021).

This balance is particularly relevant for assessing the environmental impact of dairy systems, as it highlights the total N surplus or deficit: maintaining a NB around the parity between intake and excretion is crucial for minimizing N losses to the environment. For example, in systems where N intake significantly exceeds N requirements, excess N is excreted as waste, leading to potential environmental contamination.

In order to calculate total fecal and urinary outputs, total fecal and urine collection are widely regarded as the gold standard; it is not always possible to operate in the ideal scientific environment, and therefore it is often true that alternative methos to obtain similar outcomes need to be employed.

With this idea, daily fecal excretion can be calculated by means of fecal marker, where the concept is that a good undigestible marker analyzed in the feed, is also expect to be found in the feces: therefore, if the ingested markers multiplied by dry matter intake (DMI) should be the same as the excreted marker multiplied for total fecal excretion, the latter is potentially obtained as the ratio between the marker in the feed on the feces, multiplied by DMI. Sampling protocol may vary, and it has been proved that single spot sampling of uNDF is not as reliable as using AIA (Guerra et al., 2024; Thonney et al., 1985).

Similarly, considering daily urinary excretion, indirect methods like urine spot sampling are often employed under practical constraints, such as in grazing herds or experimental conditions, where collecting funnel are not possible. Although less accurate than total collection, spot sampling provides an estimate of urine output using an analytical formula: this approach calculates total urine output by multiplying the body weight of the animal by a fixed coefficient (mg/kg BW), and then dividing by the creatinine concentration in the urine sample (mg/L) (Valadares et al., 1999). Sampling protocols may vary, with some studies using a single 6–8 hour post-feeding sample, and others may use multiple samples over several days (Boudra et al., 2022; Lee et al., 2019).

Creatinine-based estimates of urinary output provide a practical alternative to total collection, allowing for feasible N management in dairy operations, even under conditions where traditional collection methods are impractical.

While NUE focuses on the efficiency of N utilization, NB provides a holistic perspective on N flux within the system. By leveraging both NUE and NB, dairy producers can develop targeted strategies that optimize N intake relative to cows' requirements, ultimately minimizing N excretions and reducing the overall environmental impact of dairy operations.

The aim of this work is to briefly propose a picture of N balance of dairy cows in the Parmigiano Reggiano area, focusing more onto the potential technical bias that can come from the inappropriate use of the markers used to estimate total fecal and urinary output, because, in the words of Prof. Michale E. Van Amburgh "Your science is as good as your markers are".

Materials and Methods

Twenty herds, ranging from 60 to 367 lactating cows per herd, were selected from the Emilia Romagna region, known for Parmigiano Reggiano production. These herds were provided with a hay-based total mixed ration (TMR), as required by the DOP regulations. The average milk yield (MY) across herds was 34 ± 3 kg/day, with a calculated DMI of 26 ± 1.7 kg/day and an average BW of 678 ± 33 kg. Samples for fecal and urinary N analysis were collected from 10% of lactating cows in each herd, as well as individual weight. The sampling schedule spanned three days: fecal samples were collected four times across three days, while individual urine samples were taken concurrently with the fecal collections at 8 am and 2 pm on day two. Two milk bulk samples were collected in the same sampling period.

Both TMR and faecal samples were oven-dried at 55°C until they reached a constant weight, then ground using a Retch SK mill (Bauknecht, Stuttgart, Germany) to pass through a 1-mm screen. Dry matter (DM), ash, and ether extract (EE) were analyzed according to European Commission Regulation No. 152/2009. Amylase-treated neutral detergent fiber without residual ash (aNDFom),

acid detergent fiber without residual ash (ADFom), and acid detergent lignin (ADL) were analyzed using heat-stable amylase and corrected for ash (Mertens et al., 2002). Starch content was determined enzymatically (method 2014.10; AOAC International, 2014). The undigested neutral detergent fiber (uNDF) content was determined through 240-hour fermentation (Raffrenato et al., 2018) in an in vitro batch system, using rumen fluid from four cows, as described by Simoni et al. (2020); uNDF was then employed to determine fecal N output, as the product of the ratio between the uNDF content in forages and in fecaes, and DMI. Urine samples were collected via perineal massage, after losing the first drops. Each sample was then acidified immediately with 50% sulfuric acid (H_2SO_4, 1 mL in 30 mL of urine) to achieve a pH below 2; this critical step will be further discussed in the technical annex at the end of this chapter. Acidified samples were pooled by herd and analyzed for creatinine content: a visual inspection of appearance and color was conducted to ensure samples were free from contamination or alterations, such as hemolysis. Subsequently, 5 mL of urine was transferred into conical tubes and centrifuged at 500 × g at 8 °C for 10 minutes to ensure sample homogeneity. Creatinine levels were determined using an automated chemistry analyzer (BT3500, Biotecnica Instruments, Rome, Italy) based on the Jaffe method. The creatinine concentration served as a marker to estimate total urine output, allowing for an indirect assessment of urinary N excretion.

The content of N of TMR, feaces, urines and milk were measured by combustion digestion at 900°C in excess oxygen using a Dumatherm (Gerhardt GmbH & Co, Königswinter, Germany) as described by Mihaljev et al. (2015).

Results and Discussion

The diet across the studied herds was predominantly hay-based, formulated to align with the requirements of Parmigiano Reggiano production. This total mixed ration (TMR) diet included a variety of forages, with an average forage inclusion rate of 60%. The daily N intake averaged 630 g, considering a dietary CP content which ranged from 14.1% to 17.8%. These values reflect the

reliance on hay as a primary forage source, which tends to offer moderate protein levels but can also lead to significant N excretion due to its lower digestibility and higher NDIN content compared to other forage based (e.g. silages) systems (Simoni et al., 2022).

The predominant forages in the TMR were mix hay (35% of total forage content, CP 10.4 %), alfalfa hay (15% of total forage content, CP 16.57 %), and wheat hay (10% of total forage content, CP 5.46 %). These dietary characteristics are reflected in the N excretion data, where higher fecal nitrogen outputs were observed in herds with greater inclusions of forages high in uNDF. This suggests that while hay-based diets can contribute with valuable fiber, they may also lead to inefficiencies in N utilization, as a larger proportion of ingested N from forages is excreted in feces. In this study, urinary N represented a significant pathway for N excretion, ranging from 191 to 423.4 g/day, with a mean value of 272 ± 57 g/day. The most recent and comprehensive meta-analysis on this topic, which evaluated 86 studies and 307 different diets, reports an average urinary N excretion of 186 ± 61 g/day (Spanghero and Kowalski, 2021).

These differences merit specific discussion, as they directly reflect variations in urinary volume. Since urinary excretion is estimated using creatinine—by multiplying the animal's BW by a fixed coefficient (mg/kg BW) and then dividing by the creatinine concentration in the urine sample (mg/l)—any inaccuracies in creatinine measurement or body weight estimation can lead to significant discrepancies in N excretion values. In particular, as discussed further in our technical annex I, acidifying urine samples, a common practice in the ruminant nutrition field, to prevent ammonia volatilization, has shown to influence creatinine concentration (Danese et al., 2024). In fact, acidification tends to reduce creatinine levels compared to non-acidified controls: consequently, lower creatinine concentrations yield higher estimated urinary volumes, which may result in an overestimation of N excretion through urine and thus impact overall N balance calculations. Future studies should investigate the extent to which this bias quantitatively affects total urine output and N excretion values.

Concerning Fecal N, our study resulted in a range of excretion from 128.3 to 422.4 g/day, with a mean value of 234 ± 64.3 g/day; as before, our results are higher than what reported by Spanghero and Kowalski (2021), who reports an average fecal N excretion of 192 ± 42 g/day.

As previously said before on creatinine, a similar point applies to total fecal excretion measured using uNDF as a marker. The reliability of a marker is based on its recovery rate: the more the marker is recovered from feces, the more accurate it is likely to be the prediction from the marker (Sales and Janssens, 2003). Additionally, the pattern of marker excretion must be taken into account, as uNDF excretion can vary throughout the day and requires multiple sampling points for accurate assessment (Guerra et al., 2024). This is not the case for other markers like acid-insoluble ash (AIA), which shows a more consistent excretion pattern (Thonney et al., 1985).

Finally, for a marker to be reliable, its presence in the TMR must meet certain thresholds: for indigestible NDF, (Guerra et al., 2024) reports that a minimum of 750 g/day is recommended, while for AIA, the marker should constitute at least 0.75% DM (Sales and Janssens, 2003). Failing to meet these thresholds can diminish the marker's precision, as shown in previous studies (Morris et al., 2018). Therefore, since fecal samples, in the present trial, have been taken four times in three days and uNDF dietary concentration was 20.73 % DM, uNDF can be considered a reliable marker to estimate total fecal output.

Together with that, milk N averaged 180.47 ± 14.16 g/d, which was in line with what reported by f Spanghero and Kowalski (2021),with mean values of 155 ± 34 g/d.

Overall, the NB of dairy cattle considered in our studies averaged -60.77 ± 93.10 g/d, ranging from -284.43 to 131.94 g/d: this negative NB is partially due the typical Parmigiano Reggiano necessity to consider just hay as forage in the diet, therefore with a lower ttaNDFD and a higher NDIN content compared to other forages inclusion.

Conclusions

Overall, the negative NB, averaging -60.77 ± 93.10 g/day, underscores the limitations of hay-based diets in N utilization efficiency. The typical characteristics of such diets, including high fiber and lower N digestibility, exacerbate N losses compared to systems incorporating more digestible forages. Furthermore, biases introduced through marker use, storage conditions, and body weight estimation, may have inflated the negative N balance observed in these herds. Future research should focus on optimizing forage quality and refining marker methodologies to better capture nitrogen dynamics, potentially mitigating the environmental impact of nitrogen excretion in dairy systems that heavily rely on hay.

Technical Annex I: Does acidification affect creatinine in dairy cattle?

Adapted from Danese et al. (2024).

Introduction

Creatinine is a catabolic residue of creatine metabolism, excreted by the kidney in the urine, particularly suitable to be used as a marker for the estimation of total urine output in dairy cows. This attitude relates to its spontaneous, irreversible, and non-enzymatic conversion from creatine catabolism in skeletal muscles and to excretion at a constant rate (Wyss and Kaddurah-Daouk, 2000). Moreover, creatinine is only marginally influenced by muscle mass and nutrients intake, referring in particular to proteins (Da Silva et al., 2001), non-protein nitrogen (Chizzotti et al., 2008), and non-fibre carbohydrates (Rennó et al., 2000). Additionally, creatinine concentration depends also on the body hydration status, and, therefore, it has a diurnal excretion reflecting cow-to-cow variability (Lee et al., 2019b; Megahed et al., 2019). To avoid the effect of these fluctuations on the estimation of urinary output, total urine collection is addressed, but different sampling protocols have also been developed. Total urine collection is considered the gold standard for urine studies. Conversely, urine spot sampling is an indirect measure based on an analytical equation, and therefore less accurate than total collection. However, there are certain conditions (i.e., grazing herds) which force researchers toward the use of these sampling protocols. The literature reports several different approaches to spot urine sampling, varying from a single 6–8 h post-feeding sampling (Boudra et al., 2022b) to 12 sampling events in 3 days (Lee et al., 2019b). Regardless of sampling times, total urine output is calculated as the product of the body weight of the animal considered (BW, kg) and a fixed coefficient (mg/kg BW), divided by the individual urine creatinine content (mg/L). In the formula, the fixed coefficient may vary among authors and studies (Lee et al., 2019b; Valadares et al., 1999b). The use of the latter formula for total urine output quantification is frequent in the dairy industry, specifically to measure urinary Nitrogen (N) excretion. In this application, a critical point is to avoid N losses from the sample, which can occur due to ammonia N (NH_3) volatilization; for this reason, urine samples are usually acidified immediately after the collection. A widely used method is to add inorganic acids directly to the urinary samples with the objective to drop the pH level under 2 (Chizzotti et al., 2008). This also avoids the alteration of the samples deriving from the contamination

of urine by faecal bacteria and the related metabolites, which often occurs in the field (Boudra et al., 2022b). It is hypothesized that urine acidification could impact creatinine stability, detection, or measurement due to the potential chemical alteration and physical dilution effect, which a strong acid could apply to creatinine, altering its potential as a marker of total urine output. Thus, the aim of this trial was to define if urine creatinine content measurement is affected by sample acidification.

Materials and Methods

Individual urine samples from 20 Holstein lactating dairy cows of the teaching dairy barn of Dipartimento di Scienze Medico Veterinarie, Università di Parma, were collected. The cows belonged to the same dietary group and the average group feed intake and milk yield were 26 and 29 kg/d, respectively. Due to the traditional hay-based feeding system used in the teaching dairy barn, dietary ingredients were individually sampled once when fed to the animals. Dry matter (DM) content of the dietary components was measured by drying the sample at 103 °C overnight. Neutral detergent fibre (aNDF) of the dietary components was determined using heat-stable amylase but no sodium sulphite and expressed inclusive of the residual ash (Van Soest, 1994). The N content of the dietary components was determined by the combustion digestion of the sample at 900 °C in excess of oxygen by Dumatherm (Gerhardt GmbH & Co, Königswinter, Germany), as described by (Željko A. Mihaljev et al., 2015). Crude protein (CP) was calculated from N using the fix coefficient as described in (Salo-Väänänen and Koivistoinen, 1996). The cows were fed a hay-based diet, including alfalfa (aNDF 54% of DM, CP 15% of DM), mixed hay (aNDF 52% of DM and CP 13% of DM), and a commercial complementary feedstuff (aNDF 16% of DM and CP 17% of DM). Urine sampling was performed at 10 am, 4 h after feeding, during urination induced by perineal massage: the vulva and the perineal region were wiped with a clean paper towel to remove faecal residues, and the first millilitres of urine were discharged before sampling. Each cow was sampled once and the sample was immediately divided into 3 subsamples: a first subsample was acidified to obtain a pH lower than 2 (Group 1) by means of 50% sulfuric acid (H_2SO_4, 1 mL in 30 mL of urine); a second was added with the same

volume of distilled water (1 mL in 30 mL of urine) to study the dilution effect (Group 2); and the last one was stored as is without acid or water (Group 3). Urine samples were promptly refrigerated at 4 °C and transferred for urinalysis to the Clinical Pathology Laboratory of the Ospedale Veterinario Universitario Didattico (OVUD) of Università di Parma, located 5 km apart from the herd. The samples were processed, according to quality standard procedures, within two hours from the collection. For processing, after a visual assessment of appearance and colour to verify the absence of contaminations or alteration (i.e., haemolysis) of the samples, 5 mL of urine was transferred into conical tubes. Tubes were centrifugated at 500 × *g* at 8 °C for 10 min to avoid inhomogeneity of the sample. Chemical analysis and urine specific gravity (USG) were assessed on supernatant. Creatinine analyses were conducted using an automated chemistry analyser (BT3500 Biotecnica Instruments, Rome, Italy) with a specific reagent through the Jaffe method. The density of the samples was evaluated through USG by means of a hand refractometer (American Optical, Buffalo, NY, USA) to evaluate the dilution effect.

Statistical analyses were performed using Medcalc (MedCalc Statistical Software version 18.10.2; Ostend, Belgium). Normality was assessed graphically and by using the Shapiro–Wilk test. Since creatinine and USG values were not normally distributed, the data were expressed as median and range (minimum–maximum value). The Friedman test was used to compare creatinine and USG between groups for treatments (Group 1 vs. Group 2 vs. Group 3). A Bland–Altman test was applied to calculate the agreement between Group 1 and Group 3 for creatinine and 95% limits of agreement were calculated. Furthermore, a linear regression analysis was performed to describe the relationship between creatinine in Group 1 and creatinine in Group 3. The statistical significance was set at $p \leq 0.05$.

Results and Discussion

Urinary creatinine values were statistically different ($p < 0.001$) between Group 1 (median 48.5 mg/dL; range 36.9–83 mg/dL), Group 2 (median 47.5 mg/dL; range 36.5–80.7 mg/dL), and Group 3 (median 48.9 mg/dL, range 37.2–84; Figure 1). The USG resulted different between groups ($p < 0.001$), with a median of 1026 in Group 1 (range 1022–1031), a median of 1025 for Group 2 (range 1020–1030), and a median of 1025 for Group 3 (range 1022–1031). The Bland–Altman analysis showed agreement between the creatinine measured between Group 3 and Group 1 (Figure 2). The mean difference (95% CI) between creatinine measured in Group 3 and Group 1 was 0.48 (0.04 to 0.93) and the upper and lower limits of agreement were 2.35 and −1.4, respectively. The regression showed a strong linear relationship between creatinine in Group 1 and in Group 3 (R^2 of 0.99, intercept 1.017, slope of 0.97, and $p < 0.001$).

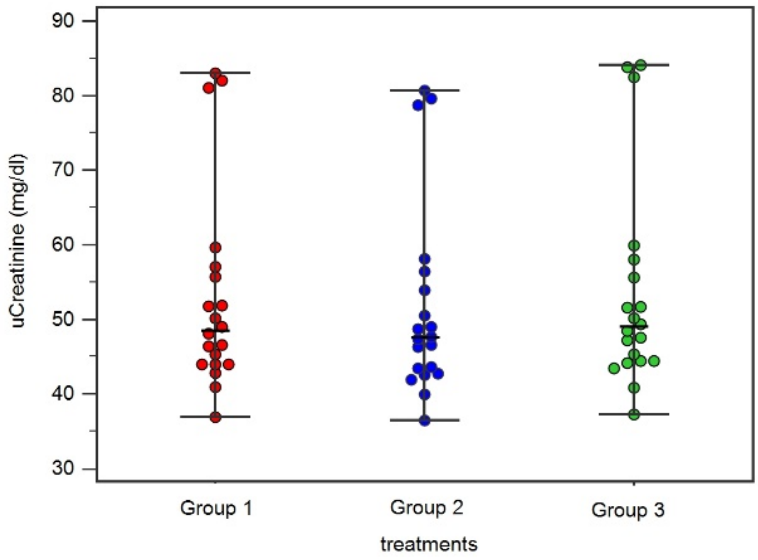


Figure 1. Dot plot showing results of urinary creatinine comparison between acidified urine (Group 1), diluted urine (Group 2), and urine without any acid or water treatment (Group 3). Upright bars represent minimum and maximum values, while horizontal lines (central bars) represent median value.

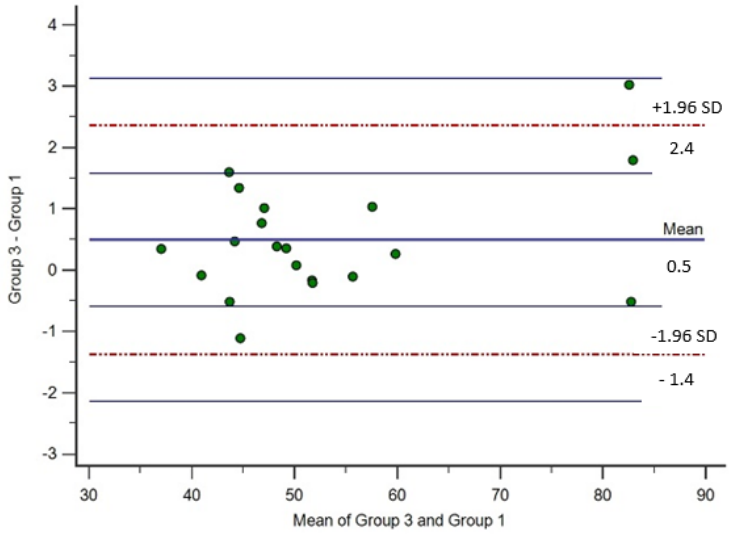


Figure 2. Limits of agreement (Bland–Altman) plot showing differences concerning urinary creatinine measured in acidified urine (Group 1) and in non-acidified urine (Group 3).

Creatinine in blood is formed by the degradation of creatine phosphate, an important energy reserve in muscle tissue metabolism. After its formation, it is filtered and excreted by the kidneys through the urine (Salo et al., 1970). Creatinine is widely used as a marker for the measurement of urinary volume through spot sampling protocols (Valadares et al., 1999b). Through the estimation of urine volume, it is possible to assess the excretion of dietary metabolites and components, of which N and purine derivatives are the main examples. Thus, an accurate measurement of creatinine in urine is fundamental for the estimation of other parameters.

Volatilization of NH_3 depends on N urea in 60 to 90% of cases, but also to a lesser extent on other important N compounds such as hippuric acids, allantoin, uric acid, xanthine, and creatinine. In order to avoid this, the acidification of samples is used for research purposes in dairy nutrition (Hristov et al., 2011).

Furthermore, sample preservation plays a central role in the measurement of creatinine in acidified urine. In fact, acidified urine preserved without freezing, especially at room temperature, can increase the conversion of creatine into creatinine (Van Niekerk et al., 1963). A recent study demonstrated that storing samples at temperatures that promoted urine freezing (−20 °C and −40 °C) is the best solution

when creatinine analysis is not performed within 15 days (da Silva et al., 2023); for this reason, samples were refrigerated and analysed within two hours of collection. Considering our study outcomes, both acidified samples (Group 1) and diluted samples (Group 2) demonstrated creatinine values lower than the non-acidified ones (Group 3). This observation leads to the hypothesis that a dilution effect was induced by the volumes introduced during the experimental procedures. Consequently, the experimental conditions, notably the acidification and dilution processes, influenced creatinine levels. In contrast, the acidified samples (Group 1) had a higher concentration of creatinine compared to the diluted samples (Group 2). This variation in creatinine levels could be related to the acid's impact on the conversion of creatine to creatinine. Despite this distinction, even with a difference observed between the acidified (Group 1) and diluted (Group 3) samples, the creatinine levels in the acidified samples (Group 1) remained lower than those in the non-acidified samples (Group 3). It is important to recognize that the implications of our findings, while possessing analytical and statistical significance, necessitate contextualization within a broader biological framework. It is considered that this could marginally impact the calculation of total urine volume output. However, such considerations do not exclude the utilization of creatinine as a reliable marker for total urine output. Further investigations and a better comprehension of these dynamics are needed to enhance the practical applicability of our findings in a field context.

Our results show satisfactory agreement between the results of creatinine analysis performed on acidified and non-acidified samples (Group 1 vs. Group 3). The slight average discrepancy between the methods confirms the statistical difference of our results.

The regression showed a strong linear relationship between creatinine in acidified samples (Group 1) and non-acidified ones (Group 3). Even if there is a statistical difference between the treatments reported and thus the data are not comparable, in the case of the use of acidified urines, it is likely that the addition of acid will homogenously act on the samples.

Thus, for the biological, nutritional, and environmental purposes of our work, we still consider creatine in acidified urines as a valuable marker for total urine output quantification when spot

sampling is performed and comparison between groups is the main goal of the study. However, the potential bias this work has revealed must be considered.

Our study has several limitations. Firstly, there is a low number of samples included for each group. Additionally, we did not conduct multiple measurements on the same sample, which would have enabled us to further explore the significance of the differences found. Nevertheless, we believe that our brief communication lays the foundation for further studies and draws attention to the importance of exploring the role of acidification in samples intended for livestock measurements.

What we reported in this communication is to be considered as preliminary data. It will be necessary to include a higher number of observations and apply an intra- and inter-assay trial to further confirm our findings.

Conclusions

In conclusion, the practice of acidifying urine to avoid N volatilization can be considered useful for environmental and nutritional evaluation purposes, but further considerations need to be made since the effect of acidification or dilution on creatinine cannot be completely excluded.

Technical Annex II: In vitro method to assess Nitrogen and dry matter total trac apparent digestibility for rumen protected amino acids

Adapted from Danese et al., 2024), and presented at the American Dairy Science Association in West Palm Beach, FL, USA.

Introduction

The principle of the 3Rs (Replacement, Reduction, Refinement) in animal science research aims to minimize the use of animals by promoting alternative methods, such as *in vitro* digestibility models that simulate digestion processes in the laboratory. This approach reduces the number of animals needed and refines experimental techniques to limit animal suffering, while still obtaining accurate data on nutrient absorption and digestive efficiency (Russel and Burch, 1960).

In the dairy nutrition environment, the principle of the 3Rs in widely employed, and allows to test *in vitro* different dietary inclusions as well as technological characteristics of the studied nutrients. Particular attention is given to amino acids, amongst which methionine (Met), which is an essential amino acid for ruminants, vital for milk protein synthesis and overall animal growth, stands out as especially important (Overton et al., 1996). Despite the rumen's ability to synthesize microbial protein, dietary supplementation of Met remains necessary due to the limited availability of certain essential amino acids like Met and Lysine (Lys) in common feedstuffs (Feng et al., 2018).

The significance of encapsulating Met lies in its ability to resist ruminal degradation while ensuring release and absorption in the small intestine, thereby optimizing the amino acid's bioavailability, and minimizing nitrogen (N) waste (Schwab, 2011).

This study aims to evaluate a novel chitosan-based biopolymer (CH) as an alternative encapsulating agent for methionine, compared to a 2-vinyl pyridine/styrene encapsulation (VP): CH is selected not only for its biodegradability but also for its potential to maintain ruminal stability and targeted intestinal release of Met.

Specifically, we wanted to characterize the novel biopolymer-encapsulated Met for its environmental and nutritional performance compared to traditional synthetic polymers: we will focus on the in vitro digestibility part and the total tract apparent dry matter (ttaDMD) and CP (ttaCPD) digestibility.

Materials and Methods

This trial was composed of different step to have a broadest view on CH: for the sake of this thesis, we will focus more on the part that was done in our lab and therefore of our interest, namely the in vitro degradability assessment.

Biodegradation Testing

To assess the biodegradability of the polymers, biological oxygen demand (BOD) was measured over 20 days, and theoretical oxygen demand (ThOD) was estimated based on chemical composition. Using these values, biodegradation coefficients (BioDeg) for both CH and VP were calculated.

Rumen Incubation Study

A rumen batch culture experiment was conducted to observe structural integrity over time. Samples of CH and VP were incubated in rumen fluid and collected at intervals of 0, 2, 4, 6, 8, 10, and 12 hours. Structural changes were analyzed using microscopy and Fourier-transform infrared (FTIR) spectroscopy, with a focus on whole spectra as well as -COOH and -NH2 functional groups.

In Vitro Degradability Assessment

For the purpose of this study, CH (n=30), VP (n=30), blank (n=6) and laboratory standard (n=6) samples were subjected to in vitro ruminal and intestinal digestion (Ross, 2013). Degradability of dry matter (DM) and crude protein (CO) was measured at 0, 4, 8, 12, and 24 hours of rumen incubation, followed by total tract degradability assessments after 12 hours of rumen incubation and 24 hours of enzymatic digestion.

Briefly, half-gram samples of the encapsulated Met were placed into 125-ml flask. Rumen fluid was collected as described in Simoni et al. (2021)and mixed with a buffered medium to simulate rumen conditions. The fluid was then added to the flasks containing the sample for incubation, conducted

in an *in vitro* batch system described by Goering and Van Soest (1970) with the modifications reported by Righi et al. (2009).

The samples were regularly shaken to enhance the contact between the polymer and microbial content. To simulate intestinal digestion, a set of samples post 12h incubation, were subjected to enzyme treatments in stages. Initially, samples were acidified to mimic abomasal conditions and then incubated with pepsin. Post-acid treatment, samples were adjusted to neutral pH and incubated with pancreatin in a potassium phosphate buffer to represent intestinal enzymatic conditions. This step simulated the breakdown and availability of methionine post-ruminal fermentation (Ross, 2013). Following incubation, samples were filtered and rinsed with hot distilled water, then dried at 105°C and weighed for ttaDMD assessment. The content of N was measured by combustion digestion at 900°C in excess oxygen using a Dumatherm (Gerhardt GmbH & Co, Königswinter, Germany) as described by Mihaljev et al. (2015), and then expressed ad CP.

Digestibility rates were calculated based on the loss of DM and CP content. The degradability results were compared between CH and VP to assess relative stability and breakdown in the simulated rumen and intestinal environments. Calculations also included adjustments for ttaDMD relative to CP content to provide a more accurate assessment of degradability.

In Situ Degradability in Cows

Two ruminally and duodenally fistulated cows, located in a European partner university, were used to measure in situ ruminal and intestinal digestibility of CH. Samples were incubated in the rumen for up to 16 hours, followed by duodenal enzyme digestion using mobile bags with a mean transit time assessment.

Results and Discussion

The biodegradation analysis revealed that the CH polymer had a significantly higher biodegradation coefficient (56.7) compared to VP (0.1), indicating superior environmental degradability. In terms

of BOD and ThOD, CH demonstrated greater oxygen consumption, further suggesting its enhanced biodegradability.

Microscopy and FTIR spectroscopy analyses showed that the VP polymer degraded structurally after 4 hours, whereas CH maintained its structural integrity up to 12 hours. CH displayed higher similarity to the original spectrum, indicating greater stability during ruminal incubation, which was confirmed by lower Euclidean distance values across all spectral measures.

The in vitro ruminal and intestinal degradation tests showed that CH provided controlled degradation of both DM and CP, with total tract degradability reaching 62.2% for DM and 91.0% for CP after 12 hours in the rumen and 24 hours of enzymatic digestion. This suggests that CH-based enMET provides sustained release through the rumen and rapid digestion in the intestine.

Finally, in situ degradability tests in fistulated cows showed minimal degradation in the rumen but complete digestion post-duodenal transit, further affirming the product's stability in the rumen and availability in the intestine.

Conclusion

The chitosan-based biopolymer coating of enMET exhibits promising biodegradability, high stability in the rumen, and excellent intestinal availability. This sustainable alternative to synthetic polymers not only reduces environmental impact but also maintains effective nutrient delivery, making it a viable option for animal nutrition applications.

General Conclusions

This thesis provides a comparative analysis of different markers—uNDF, uNDFom, AIA, and partially also ADIA ad ADL–used to estimate ttaD and ttD in various ruminant species and categories, namely dairy sheep and goats, rams, dairy buffaloes, dairy cows and beef cattle. Across the trials, the results demonstrated that marker selection significantly influences digestibility estimates, with variations observed based on species, diet type, and specific nutrients analyzed, particularly in fiber-rich diets.

In trials focused on small ruminants and lactating buffaloes, uNDF consistently provided higher estimates of digestibility, particularly ttaDMD and ttNDFD, although it showed variability linked to circadian patterns and dietary composition. Conversely, AIA emerged as a reliable marker for scenarios with limited fecal sampling frequency, as it exhibited stable recovery rates in lactating sheep and goats, even if . For forage-based diets, such as those fed to dairy cows and beef cattle, uNDFom performed as an optimal marker for fiber digestibility due to its exclusion of ash, offering consistent and accurate results across dietary inclusions, even if still strongly related and correlated to uNDF.

In beef cattle, ADL was also shown to provide comparable digestibility to uNDF estimates, though it exhibited greater variability, making uNDF a preferable option for consistent measurements specifically considering ttaCPD. When considering the use of makers as tools to evaluated total faecal or urine output when the total collection is not feasible, namely uNDF and creatinine, they could provide reliable results when employed following literature technical limitations.

A sufficient dietary proportion of the marker within the diet, the experimental sampling protocol chosen and sampling schedule, and the storing conditions are essential to avoid alterations of the marker's concentrations.

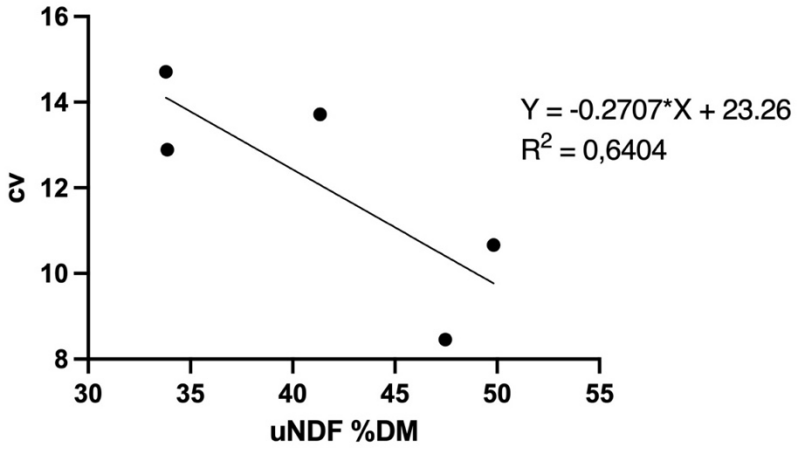
Fecal uNDF concentration as a digestibility marker: proposal insights into precision and variability through coefficient of variation analysis

Overall, an analysis of fecal uNDF concentration (x; %DM) across the trials (1 to 5)—without differentiation by animal species or diet composition—was conducted by plotting it against the respective coefficient of variation (y; CV). The relation found seems to be satisfactory described by a linear regression, having a negative slope and an R^2 value of 0.6404.

These findings suggest that as fecal uNDF concentration (%DM) increases, the CV decreases, indicating a trend for a lower variability of the data when the concentration of the faecal marker increase. Specifically, the increase in marker concentration explains 64% of the variability of the CV. The remaining 36% of the variability can be attributed to other experimental parameters, such as diet composition, sampling protocols and sample management and analytical techniques.

This regression provides a simple tool to support the theory that the more representative markers are those present in higher concentration in the feaces. Additionally, when total fecal collection—and thus the marker recovery analysis—is not feasible due to experimental constraints, this approach can help evaluate the reliability of uNDF as a digestibility marker.

Figure 1. Coefficient of variation (cv) within the population considered (y), plotted together with the average values of the concentration of the chosen markers (x; %DM).



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