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Indigestible neutral detergent fibers: Relationship between forage fragility and neutral detergent fibers digestibility in total mixed ration and some feedstuffs in dairy cattle

Mostafa Soufizadeh, Rasoul Pirmohammadi*, Yunes Alijoo, Hamed Khalilvandi Behroozyar

Department of Animal Science, Faculty of Agriculture, Urmia University, Urmia, Iran.

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Abstract

Indigestible neutral detergent fibers (iNDF) accurately predict forage digestibility when measured *in situ*. The objective of this study was to determine the effects of rumen incubation times on the estimated concentrations of iNDF for four forages (alfalfa hay, corn silage, wheat straw and orchard grass), four concentrates (barley grain, soybean meal, beet pulp and wheat bran) and two total mixed ration samples in dairy cows. The iNDF contents of the samples were evaluated in 10 feeds using three ruminally cannulated Holstein cows in a completely randomized design. Five grams of the samples were incubated up to 240 hr. The iNDF fraction was significantly affected by incubation time for all of the tested samples, but the potentially digestible NDF fraction (pdNDF) was not affected for wheat straw, barley grain and wheat bran (32.32, 10.11 and 20.60 g per 100 g of dry matter, respectively). For most of concentrates feedstuffs, the iNDF fraction could be measured after 120 hr of incubation, while for forages ruminal incubation should be lasted up to 240 hr. Statistically significant differences ($p < 0.01$) were observed between forage samples regarding fragility and NDF digestibility (NDFD). Also, a positive correlation was observed between fragility and NDFD. In some of the cases, it appears that NDFD can be a more helpful index in adjusting pdNDF values than direct fragility measurements.

الیاف گوارش‌ناپذیر نامحلول در شوینده خنثی: ارتباط بین شکنندگی علوفه و گوارش‌پذیری الیاف نامحلول در شوینده خنثی خوراک کاملاً مخلوط و برخی اقلام خوراکی در گاوهای شیری

چکیده

اندازه‌گیری الیاف گوارش ناپذیر نامحلول در شوینده خنثی (iNDF) در جایگاه طبیعی قادر به پیش بینی دقیق مقادیر گوارش پذیری علوفه می باشد. هدف این مطالعه ارزیابی آثار زمان های انکوباسیون شکمبه بر روی برآورد غلظت های iNDF برای چهار نوع خوراک علوفه ای (یونجه خشک، ذرت سیلوشده، کاه گندم و علف باغی)، چهار نوع خوراک کنسانتره‌ای (دانه جو، کنجاله سویا، تفاله چغندرقند و سبوس گندم) و دو نمونه خوراک کاملاً مخلوط در گاوهای شیری بود. مقادیر iNDF در ۱۰ خوراک با استفاده از سه رأس گاو هلشتاین کانولا گذاری شده در شکمبه در یک طرح کاملاً تصادفی مورد ارزیابی قرار گرفت. پنج گرم از نمونه ها داخل کیسه های پلی استر تا ۲۴۰ ساعت انکوبه شدند. بخش iNDF به طور معنی داری برای تمامی نمونه های آزمایشی تحت تاثیر زمان انکوباسیون قرار گرفت، اما بخش بالقوه گوارش‌پذیر NDF (pdNDF) برای کاه گندم، دانه جو و سبوس گندم (به ترتیب ۳۲/۳۲، ۱۰/۱۱ و ۲۰/۶۰ گرم در ۱۰۰ گرم ماده خشک) تحت تأثیر قرار نگرفت. برای بیشتر اقلام خوراکی کنسانتره‌ای ، بخش iNDF پس از ۱۲۰ ساعت از زمان آغاز انکوباسیون قابل اندازه گیری بود، درحالی که زمان انکوباسیون شکمبه ای برای علوفه ها باید ۲۴۰ ساعت به طول می انجامید. تفاوت های معنی داری ($p < ۰/۰۱$) بین نمونه های علوفه در رابطه با شکنندگی و گوارش پذیری NDF (NDFD) مشاهده گردید . همچنین، همبستگی مثبتی بین شکنندگی و NDFD مشاهده شد. در برخی موارد، به نظر می رسد که NDFD می تواند شاخص کارآمدتری جهت تنظیم مقادیر pdNDF نسبت به اندازه گیری های مستقیم شکنندگی باشد.

واژه های کلیدی: دیواره سلولی، شکنندگی، گوارش‌پذیری الیاف، مخازن الیاف شکمبه

***Correspondence:**

Rasoul Pirmohammadi. MSc, PhD
Department of Animal Science, Faculty of Agriculture, Urmia University, Urmia, Iran.
E-mail: r.pirmohammadi@urmia.ac.ir

Introduction

A wide range of *in vitro* and *in situ* techniques are used as alternatives to *in vivo* measurement of ruminal fiber availability. Fiber digestibility and forage fragility are critical factors should to be considered in forage evaluation and diet formulation for ruminants.[1,2] Digestive characters of dietary neutral detergent fibers (NDF) fraction have been reported to greatly affect feeding behavior, chewing activity, rate of particles breakdown, ruminal turnover rate, ruminal fill dry matter (DM) intake and overall efficiency of milk and milk components production. Traditionally, nutritionists have focused primarily on fiber digestibility parameters measures and kinetics of ruminal neutral detergent fiber (NDF) and acid detergent fiber (ADF) degradations. However, recent studies have suggested to include indigestible fiber estimates as important fiber characters with ability to influence turnover rate of ruminal fiber pools and eventually set the rate and final extent of rumen fiber digestion.[1] Accurate estimation of the potentially digestible NDF (pdNDF) fraction and its rate(s) of digestion in nutritional modeling requires precise determination of indigestible NDF (iNDF) content.[1,2] Digestibility of the fiber has been reported to be related to potentially digestible portion of NDF.[3,4] The pdNDF are defined as the difference between the NDF and iNDF fractions. Potential digestibility is repeatedly defined as the NDF fraction disappearing after a long ruminal incubation period.[5] The iNDF will not be available to microbial digestion in ruminants, even if the total tract residency of fibers extended to an infinite time.[6] Indigestibility of the iNDF can be attributed to the cross-links between cell wall lignin and hemicellulose.[7] According to Ellis *et al*., iNDF determination should be considered as an inevitable test in forage evaluation for the estimation of pdNDF.[8] Because of zero digestibility and potential effects on animal performance, it is recommended to define an upper limit for dietary iNDF in high producing of dairy cows. Lippke has suggested about 20 g kg^{-1} of metabolic weight a day as a maximum tolerable dietary iNDF consumption.[9]

Fragility is defined as a relative rate of forage particle size reduction during chewing or laboratory simulation of chewing action. Fragility has been reported to be related to digestibility, lignin content and anatomical characters such as cell wall thickness.[7]

Consequently, fragility measures of the forage cell wall can be used as predictive tools for forage digestibility. According to Minson,[10] grass forages can be classified into high (81.00%), medium (72.00%) and low (56.00%) digestible feed for sheep, based on chemical composition and resistance to comminution and voluntary intake. Additionally, Minson has reported a negative correlation between NDF digestibility (NDFD) and chewing activity.[10] However, correlation coefficient was reported to be positive between NDFD and samples type. Grant has found that the 24 hr *in vivo* NDFD explains about 60.00% of the variation in forage fragility.[11] The potential exists to combine a fragility factor related to NDFD, with the physically effective factor (pef) derived by sieving to arrive at a superior value to predict cow chewing response.[11]

Notwithstanding, there is a small, but developing data set regarding forage fragility and its relations with potential ruminal digestibility of plant cell wall. The objective of this study was to determine the effects of ruminal incubation time on iNDF concentration estimation in total mixed ration (TMR) and some of the forage and concentrate feeds in dairy cow nutrition programs. Additionally, relationships between fragility and NDFD of forage samples were addressed.

Materials and Methods

Animals, samples and chemical analysis. All of the experimental protocols were approved by the Animal Use Committee in Urmia University, Urmia, Iran (proposal No. 2606; 06.06.2016). The animals were cared according to Guide for the Care and Use of Agricultural Animals in Research and Teaching.[12] Rumen cannulated mature Holstein steers (520.00 ± 15.00 kg) were fed a TMR containing 20.00% chopped alfalfa hay, 30.00% corn silage, 25.00% wheat straw and 25.00% barely grain plus mineral/vitamin supplement according to their requirements for two times daily at 8:00 and 18:00 hr. Four concentrate feeds (barley grain, soybean meal, beet pulp and wheat bran), four forages (alfalfa hay, wheat straw, orchard grass hay and corn silage) and two TMR (the concentrate-to-forage ratio in the TMR was 45:55 on DM basis, Table 1) samples were used in this study. The effects of incubation time (96, 120 and 240 hr) on the concentrations of iNDF were evaluated using three ruminally cannulated Holstein cows in a completely randomized design. Feed samples were dried in a forced-air oven at 60 ˚C for 48 hr and ground to pass a 1.00-mm screen by a Wiley mill (Ankom[100] Fiber Analyzer; Ankom Technology, Macedon, USA) before chemical analysis.[13]

Feed samples were analyzed for DM (at 55 ˚C for 48 hr),[14] NDF, ADF and lignin.[15] Neutral detergent solution contained sodium sulphite and heat stable alpha amylase.[16] Ash content was determined by ignition of the dried samples at 500˚C for 4 hr.[13] Ash was determined in the bag residues and NDF was expressed free of residual ash. The lignin content was determined by solubilizing of the ADF fraction in 12 M sulfuric acid.[17]

Forage fragility. Fragility parameters were analyzed according to Miner Institute developed ball milling method.[18] Briefly, forage samples were dried at 60 ˚C for

Table 1. Ingredient (% of dry matter) and chemical composition (g per 100 g of DM) of experimental rations and feed samples used *in situ*.

Item	TMR1	TMR2	Alfalfa hay	Wheat straw	Orchard grass hay	Corn silage	Barley grain	Soybean meal	Beet pulp	Wheat bran
Ingredient composition										
Alfalfa hay	33.00	33.00								
Wheat straw	16.00	2.00								
Orchard grass hay	2.00	16.00								
Corn silage	4.00	4.00								
Barley grain	30.00	26.00								
Soybean meal	1.50	1.00								
Beet pulp	7.00	7.50								
Wheat bran	5.00	9.00								
Bicarbonate sodium	0.20	0.20								
Dicalcium phosphate	0.40	0.40								
Mineral / Vitamin	0.50	0.50								
Salt	0.40	0.40								
Chemical composition										
DM	72.31	72.00	91.50	93.30	92.55	32.50	91.90	90.90	92.20	89.10
Ash	5.20	5.70	8.20	7.80	9.80	3.33	2.50	6.75	6.30	6.90
NDF	35.29	36.05	44.43	73.12	56.13	45.30	19.77	13.68	41.49	43.33
aNDFom[1]	34.40	34.90	41.00	69.78	54.96	41.16	17.53	9.93	30.05	39.80
ADF	23.95	25.51	36.40	48.04	33.27	30.37	6.72	8.54	26.52	13.43
Lignin	6.75	7.10	14.13	14.23	10.30	10.88	1.57	2.37	4.31	5.65

[1] NDF with sodium sulfite, amylase and ash correction; TMR: Total mixed ration.

48 hr and placed in a ball mill loaded with ceramic balls (l-2.60 mm, milling time: 15 min at 80 rpm). The forage samples were sieved for calculation of physically effective fiber index (particles greater than 1.18 mm) prior to (pef_i) and followed milling (pef_{BM}).[11] Fragility was determined as a change in proportion of particles greater than 1.18 mm sieved by dry vertical sieving of the ball-milled forage from the original sample:

$$Fragility = \frac{pef_i - pef_{BM}}{pefi} \times 100$$

***In situ* incubations.** The iNDF concentration of each feed sample (2.00 mm screen) was determined following *in situ* incubations for up to 96, 120 and 240 hr in the rumen. All samples (5.00 g) were weighed into polyester bags (7 cm × 8 cm) with a pore size of 15.00 ± 2.00 µm and a pore area equal to 6.00% of the total surface area and incubated in duplicate within each cow.[13] Samples of the internal dimensions of the nylon bags and the sample size were adjusted to give a sample size to surface area ratio of 10 mg per cm^2. After removal, the bags were rinsed twice (for 12 min) in 25 ˚C water in a washing machine, boiled for 1 hr in neutral detergent solution including sodium sulfite (100 mL g^{-1} of sample) and thoroughly rinsed twice with boiling water.[16] The washed samples were rinsed twice with 30 mL of acetone, allowing for 2 min soak with each rinse, dried at 100 ˚C for 24 hr and weighed.[19]

The bag residues were analyzed for NDF and ash content. The iNDF content and iNDF 2.40 were calculated according to NDF content of the bag residues and 2.40 times of acid detergent lignin (ADL), respectively.[20]

The pdNDF content was calculated based on difference of total NDF and iNDF and NDFD of forages was determined based on methods outlined by Grant as follows:[11,21]

$$NDFD_t = 100 - iNDF_t$$

and

$$pdNDF_t = NDF - iNDF_t$$

Statistical analysis. The complete data set was analyzed as a completely randomized design using PROC GLM of SAS (version 9.1; SAS Institute Inc., Cary, USA). Least square means were adjusted by Tukey and separated using PDIFF option. Additionally, PROC REG was used to investigate the relationship between different measurements. Data were presented as least square means and corresponding standard errors.

Results

Chemical analysis. Chemical compositions of tested feed samples are represented in Table 2. Calculated iNDF and pdNDF values of the feed samples and TMRs are also reported in Tables 1 and 3. All of the feeds displayed a chemical composition within expected ranges. Concentrations of iNDF within each sample varied according to incubation time ($p < 0.01$). Generally, iNDF was higher in forage than concentrate samples. The TMR rations were contained different ingredient but formulated to have similar chemical composition (Table 2). The range for lignin concentration was very wide from 1.57 to 14.23% of DM for barley and wheat straw, respectively.

Table 2. The indigestible NDF (iNDF g per 100 g of DM) and potentially digestible portion of NDF (pdNDFg per 100 g of DM) of feed samples after 96, 120, and 240 hr *in situ* incubation and estimation of indigestible fiber by lignin × 2.40 ($iNDF_{2.40}$).

Item	Alfalfa hay	Wheat straw	Orchard grass hay	Corn silage	Barley grain	Soybean meal	Beet pulp	Wheat bran	Experimental ration		SEM	*p*-value
									TMR1	TMR2		
$iNDF_{96}$	28.77^{c}	45.01^{a}	34.15^{b}	34.28^{b}	12.30^{e}	3.82^{f}	24.40^{cd}	25.74^{cd}	24.10^{cd}	22.76^{d}	7.321	<.0001
$iNDF_{120}$	24.62^{c}	45.32^{a}	29.05^{b}	28.73^{b}	9.56^{e}	0.84^{f}	19.41^{d}	23.03^{c}	17.95^{d}	18.23^{d}	1.478	<.0001
$iNDF_{240}$	22.27^{cd}	40.80^{a}	26.09^{b}	24.01^{bc}	9.65^{g}	0.83^{h}	19.76^{de}	22.72^{cd}	17.83^{ef}	16.25^{f}	3.334	<.0001
$iNDF_{2.40}$	33.92^{a}	34.16^{a}	24.72^{b}	26.12^{b}	3.77^{f}	5.68^{f}	10.34^{e}	13.56^{d}	16.20^{cd}	17.04^{c}	2.428	<.0001
pdNDF												
96 hr	15.66^{bcd}	28.11^{a}	21.98^{b}	11.01^{cde}	7.46^{e}	9.86^{de}	17.09^{bc}	17.59^{bc}	11.18^{cde}	13.88^{cde}	12.00	<.0001
120 hr	19.81^{bc}	27.79^{a}	27.08^{a}	16.56^{c}	10.21^{d}	12.84^{d}	22.07^{b}	20.29^{bc}	17.33^{c}	18.41^{bc}	4.307	<.0001
240 hr	22.16^{b}	32.32^{a}	30.04^{a}	21.28^{b}	10.11^{d}	12.85^{d}	21.72^{b}	20.60^{bc}	17.46^{c}	20.39^{bc}	3.091	<.0001

Means within a row with different superscripts differ significantly at $p < 0.05$.

Table 3. The indigestible NDF (iNDFg per 100 g of DM) residues and the potentially digestible portion of NDF (pdNDF g per 100 g of DM) of feeds after 96, 120, and 240 hr i*n situ* incubation.

Feed	Time (hr)			SEM	*p*-value
	96	120	240		
iNDF					
Alfalfa hay	28.77^{a}	24.62^{b}	22.27^{c}	0.86	< 0.01
Wheat straw	45.01	45.32	40.80	20.52	0.44
Orchard grass hay	34.15^{a}	29.05^{b}	26.09^{c}	2.72	< 0.01
Corn silage	34.28^{a}	28.73^{b}	24.01^{b}	6.39	< 0.01
Barley grain	12.30^{a}	9.56^{b}	9.56^{b}	0.64	< 0.01
Soybean meal	3.82^{a}	0.84^{b}	0.83^{b}	0.02	< 0.01
Beet pulp	24.40^{a}	19.41^{b}	19.76^{b}	1.07	< 0.01
Wheat bran	25.74	23.03	22.72	6.75	0.35
Experimental rations					
TMR1	24.10^{a}	17.95^{b}	17.83^{b}	0.90	< 0.01
TMR2	22.76^{a}	18.23^{b}	16.25^{c}	0.51	< 0.01
pdNDF					
Alfalfa hay	15.66^{c}	19.81^{b}	22.16^{a}	0.46	< 0.01
Wheat straw	28.11	27.79	32.32	18.67	0.41
Orchard grass hay	21.98^{c}	27.08 ^{b}	30.04^{a}	2.05	< 0.01
Corn silage	11.01^{b}	16.56^{ab}	21.28^{a}	22.07	< 0.01
Barley grain	7.46	10.21	10.11	4.04	0.24
Soybean meal	9.86^{b}	12.84^{a}	12.85^{a}	1.20	< 0.02
Beet pulp	17.09^{b}	22.07^{a}	21.72^{a}	3.73	< 0.03
Wheat bran	17.59	20.29	20.60	7.79	0.40
Experimental rations					
TMR1	11.18^{b}	17.33^{a}	17.46^{a}	2.18	< 0.01
TMR2	13.88^{b}	18.41^{a}	20.39^{a}	2.44	< 0.01

Means within a row with different superscripts differ significantly at ($p < 0.05$).

The highest difference in iNDF concentration was for wheat straw and soybean meal measured after 120 hr of incubation (45.32 and 0.84% DM; $p < 0.01$, respectively).

Effect of incubation time on iNDF and pdNDF. With the exception of wheat straw and wheat bran, increasing in incubation time decreased ($p < 0.05$) iNDF concentrations of forages, concentrate feeds and TMR (Table 3). Results showed that for the most of the concentrates, the iNDF concentration can be measured after 120 hr incubation, while for forage feeds, ruminal incubation should be lasted up to 240 hr. Calculated values for iNDF 2.40 in alfalfa hay and soybean meal were greater than measured iNDF after different incubation times (Fig. 1A, $p < 0.05$). However, in orchard grass hay, barley grain and beet pulp the measured values were greater than estimated values using lignin content.

The values for pdNDF are presented in Table 3. As shown, pdNDF in alfalfa hay and orchard grass hay had higher ($p < 0.01$) digestion rates than corn silage (at 96 hr incubation time). In the case of other feeds, pdNDF rates were slow. However, digestion rate for potentially digestible fraction of NDF can have a big impact on ruminal digestion extent. The pdNDF digestibility of alfalfa hay was the same as corn silage one (at 240 hr incubation) alongside with a quite different process. In corn silage, larger fractions of potentially digestible fiber digest slowly, but in the case of alfalfa hay, a smaller proportion of potentially digestible fiber accompanied with higher digestion was found compensating greater iNDF pool.

Fragility and NDF digestibility. Physical affectivity of fiber was shown to be affected by ball milling (Table 4). The fragility indices in alfalfa hay and wheat straw were higher and lower than other forage samples, respectively (49.49% of DM versus 13.98% of DM). Orchard grass hay had lower fragility than alfalfa hay and corn silage ($p < 0.01$). This result confirmed the reduction of fragility as a function of particle size. In the case of orchard grass, it had shown a greater impact than alfalfa hay and corn silage. The NDFD coefficient after 240 hr of ruminal incubation was greater for alfalfa hay than other forage samples. The *in situ* 240-hr NDFD for wheat straw was 59.19% compared to 77.72% of DM for alfalfa hay ($p < 0.01$) and averaged 74.94% of DM for orchard grass hay and corn silage (Table 4).

Figure 2 shows the regression line between fragility and NDFD after 96, 120 and 240 hr of incubation, respectively. There is a relationship between fragility and NDFD up to a certain point, perhaps ~55.00% of fragility. The R^2 indicates that the fragility explains about 55.00% of the variation in NDFD. However, beyond this point, there is no relationship between fragility and NDFD. It does make sense that above certain fragility, greater fragility results in no further enhancement in NDFD.

Table 4. Fragility and NDF digestibility (NDFD as % of dry matter) of forages after 96, 120 and 240 hr *in situ* incubations.

Item	Alfalfa hay	Wheat straw	Orchard grass hay	Corn silage	SEM	*p*-value
Fragility (%)						
Initial physical effective factor	88.93[b]	95.06[a]	82.76[c]	96.63[a]	0.743	< 0.0001
After milling physical effective factor	44.93[d]	81.76[a]	63.56[c]	70.33[b]	0.410	< 0.0001
Fragility index	49.46[a]	13.98[d]	23.19[c]	27.21[b]	0.990	< 0.0001
Un-fragility	50.53[d]	86.01[a]	76.81[b]	72.78[c]	0.990	< 0.0001
NDFD (%)						
96 hr	71.22[a]	54.98[b]	65.84[a]	65.71[a]	15.063	< 0.005
120 hr	75.37[a]	54.67[c]	70.94[b]	71.26[b]	1.223	< 0.0001
240 hr	77.72[a]	59.19[b]	73.90[a]	75.98[a]	6.600	< 0.0001

Means within a row with different superscripts differ ($p < 0.05$).

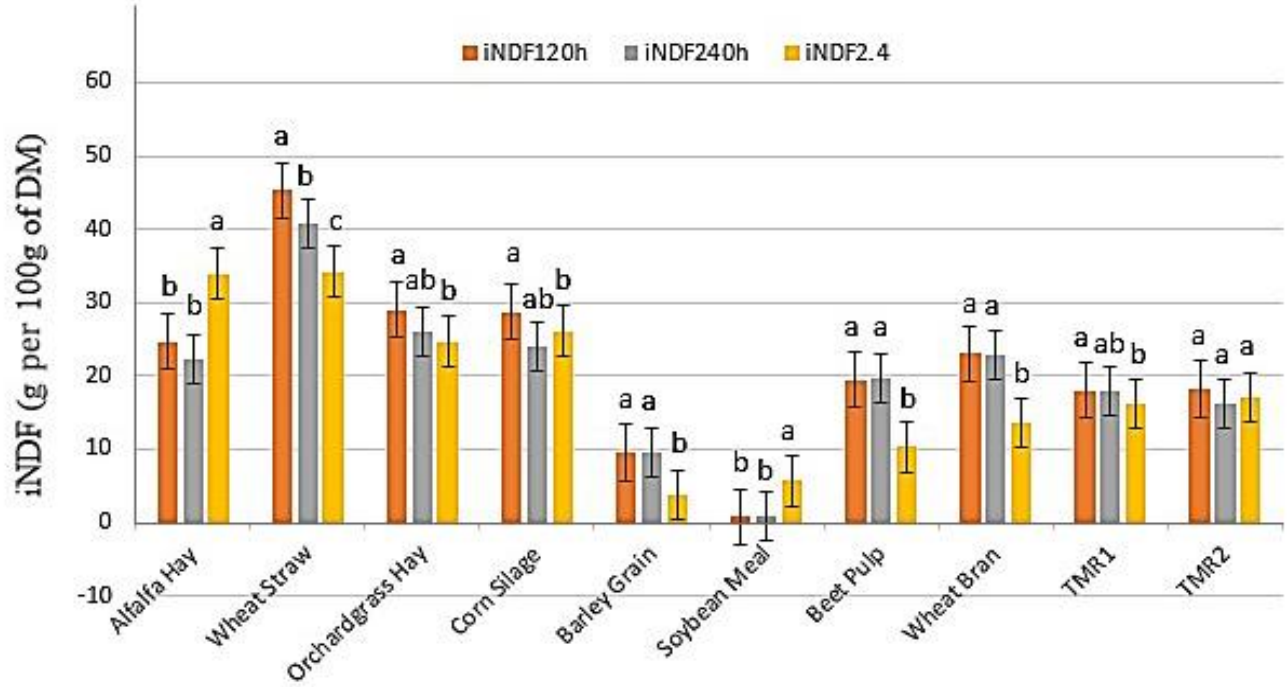

Fig. 1. Comparisons between different values of iNDF concentration of feed samples at 120 hr and 240 hr *in situ* incubation times and estimation of indigestible fiber by lignin × 2.40 ($iNDF_{2.40}$).
Mean values with different letters are significantly different at ($p < 0.05$).

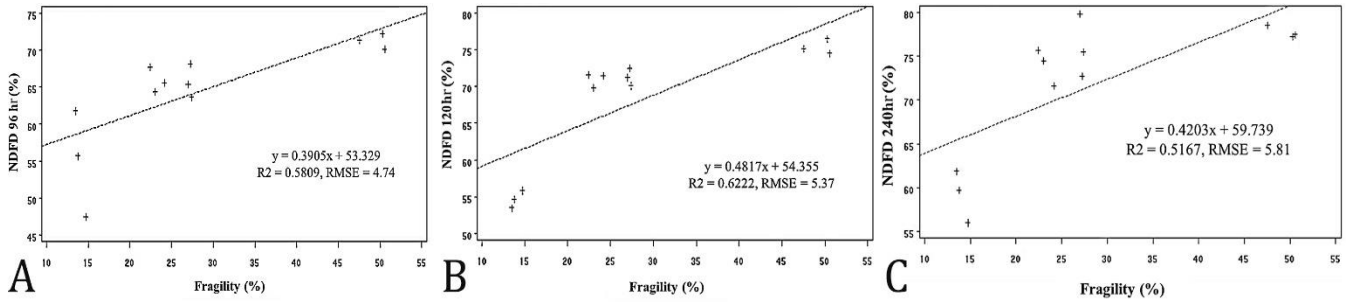

Fig. 2. Relationship of the 96-hr (A), 120-hr (B) and 240-hr (C) *in situ* NDF digestibility of forages with fragility index of the forages as measured by change in physical effectiveness factor following ball milling.

Discussion

Within tested samples, forages had higher NDF and slightly higher iNDF contents than concentrate sources (Tables 1 and 2). In wheat straw and corn silage, the NDF are mainly concentrated in vascular tissues of leaves and stems. Progressing with maturity, NDF are increasingly lignified and the digestibility is declined.[10] In alfalfa hay and orchard grass, the NDF are likewise concentrated in vascular tissues of stems and only to small amounts in the leaves. Higher leaf to stem ratio and low NDF content of the leaves resulted in higher forage fragility (Table 4). The

NDFD was reported to be a function of various factors including forage species, maturity stage, number of harvest, latitude and climate.[10] Van Soest has outlined that the nature and extent of lignification in forage cell walls control NDF.[7] The results showed that lignin cannot be an accurate estimator for iNDF or pdNDF contents, because lignin is not an uniform chemical entity of the forage cell wall.[1,2] This results were confirmed by other studies[2,6,13] attempted to describe that forage iNDF from lignin contents generally have low accuracy and precision. Reaching a point where the sample residue weight does not change significantly with additional hr of fermentation is a measure of uNDF. As iNDF influence ruminal retention, digestion and passage dynamics and physical effectiveness of the fiber, they can be used for effective estimation of digestion rates and extent.[22]

As ruminal retention time and potential digestion extent of forage cell wall are the most important limiting factors for high-producing animals, understanding digestion kinetics will be inevitable. Several reports have described iNDF and their important roles in reliable determination of digestion kinetics as well as their use as internal markers.[1,13,22] However, measures of uNDF (what has not been digested after a specified amount of time), typically were used to estimate iNDF. According to the Lucas principle,[23] iNDF are digested at a predictable rate of zero. Precise estimates of the fiber kinetic parameters, need accounting on a correctly estimated iNDF residue.[22] However, there are very limited data about iNDF fractions and potential impacts on ruminal fiber digestion. In some of the cases, iNDF values determined over a too-short fermentation time will use.[24] In this study, increasing the *in situ* incubation time from 96 to 240 hr significantly reduced the measured iNDF concentration for all of feed samples. Results of this study showed that iNDF content of concentrate can be measured after 120 hr. Nevertheless, forage samples need longer incubation time up to 240 hr for a reliable iNDF estimation,[1,13] because lignin content in forages and concentrate samples is different. As suggested before, forage NDF behave in a heterogeneous manner with two distinct digestible pools and the undigested NDF (uNDF) determined from a 240 hr *in vitro* digestion.[22,24] It has been documented that extending the incubation from 120 to 240 hr, reduces measured iNDF by 5.00 to 15.00%.[25,26] In this case, the asymptotic values estimated from a nonlinear model of 96-hr *in situ* disappearance data did not give precise predictions of iNDF compared to the results of the 288-hr ruminal *in situ* incubation.[27,28] Moreover, measurements of pdNDF in grass and clover forages based on ruminal *in situ* incubation times of up to 168 hr were substantially lower than those obtained from 504 hr of incubation.[29] It has been observed that pdNDF determined at the 240-hr incubation time were higher than other incubation times for all of the samples. It was suggested that this may occur due to digestion rate reduction over extended incubations.[13] Mertens has recommended that the asymptotes can only be accurately estimated when digestion is > 99.00% complete, which is rarely the case for most of the *in situ* incubations of 96 hr or less.[30] Digestion of NDF was reported to continue even after long incubation periods,[31] proposing that extended incubations are required to precise estimation of iNDF. The present study may suggest that when small pore size bags were used, ruminal incubations for at least 120 hr for concentrates and 240 hr for forage samples are obligatory. In agreement with our *in situ* data, Van Soest *et al.* have suggested that forages have a fast- and a slow-digesting fiber pool *in vitro*, with digestion rates of 0.40 to 1.20% per hr for slow digesting pool.[32]

In this study, for accurate estimation of the values of iNDF and pdNDF of feeds, the values obtained at different time *in situ* incubation (Tables 2 and 3), because a single time point assay is not a direct measure of iNDF and pdNDF or rate of fiber digestion. Lopes *et al.* have suggested that the use of a single-time point incubation to predict NDFD is not adequate.[19] A single-time point *in vitro* NDFD assay simply indicates how much residual fiber remains after a specific period of exposure to rumen fluid. The measured residue includes not only the indigestible fiber fraction, but also potentially digestible fiber did not degrade. Lopes *et al.,* have showed that for each percentage unit increase in iNDF content, 0.96 percentage units reduction in total-tract NDFD can be expected.[19] This relationship confirms the importance of an accurate iNDF measurement in forage evaluation and to NDFD prediction models development.[13] Huhtanen *et al.* have noted that a moral model of NDFD estimation has to separate NDF into indigestible and potentially digestible fractions.[33]

In the present study, all of the samples were analyzed for lignin by solubilization of cellulose in 12 M sulfuric acid after extraction with acid detergent according to the procedure described by Gomes *et al.,* and iNDF were estimated by long-term rumen incubation and 2.40 folds of the lignin content.[17] However, the measured *in situ* values at 120 and 240 hr were significantly different (Fig. 1, $p < 0.05$) compared to those predicted by iNDF 2.40. These differences between measured and calculated can potentially bias rate and extent of NDFD and dietary energy predictions.[22] Lower accuracy of iNDF 2.40 values in the present study is in line with other studies tried to predict forage iNDF fraction via ADL content.[7,34] Huhtanen *et al.* have revealed that the fixed relationship between lignin and iNDF is not fitting and cannot accurately describe the digestible pool of NDF in all of the feed classes.[33] Generally, lignin has been regarded as a primarily limiting factor in forage digestion. Van Soest *et al.* have confirmed the linear relationship ($R^2 = 0.94$, iNDF = 1.89) between lignin and iNDF for various forage sources.[32] Despite the biological relevance of using lignin in iNDF predictions, it has been shown to be unsuccessful

when compared across the years and forage types.[7,35] Even though, the 2.40× as a predictive coefficient was confirmed between permanganate lignin and iNDF by Huhtanen *et al*.[6] This index for individual forage species (primary and regrown grasses, red clover and whole-crop cereals) varied between 2.80 and 5.50.[6] Krämer *et al.* have observed an even greater range (from 0.30 to 4.70) for the relationship between iNDF and acid detergent lignin ADL when concentrates and byproducts were evaluated alongside different types of forages.[35]

In addition to inconsistent inter- and intra-laboratorial lignin analysis results, lignin is not a uniform entity of the cell walls.[7] Unpredictable relationship between lignin and indigestible NDF fraction, can be attributed to variances in cross-links of the lignin and cell wall carbohydrates among different forage species and maturity stages.[7] Different factors such as adopted methods for ADL, iNDF and non-lignin characteristics of cell walls were reported to affect NDF indigestibility estimates.[23] Furthermore, different environmental factors such as temperature and light intensity have been reported to affect relationship between lignin and cell wall carbohydrates.[6] In a recent study using tropical forages,[17] lignin analyzed by several methods was significantly correlated with iNDF concentration, but the resulting prediction errors were relatively high (58.70 to 87.30 g kg^{-1} of NDF). This may relatively reflect high errors in the determinations of iNDF and lignin, despite protein contamination correction of the latter. As a result, iNDF estimations based on lignin content of feed cannot be reasonably estimated.

In this experiment, the fragility indices of the forage samples via ball milling were measured and their relationships with NDFD were assessed. Fragility index as an important factor has been reported to influence chewing response of dairy cattle.[11] A fragility index of 0, reveals very tough sample, with no reduction of particle size upon ball milling and a 100 percent index is parallel with complete particle size reduction to less than 1.18 mm, $pe_{fi} = pef_{BM}$.[11] As shown in Table 4, there were significant differences in forages fragility as measured by changing in particle size. This can be a hint to accounting forage fragility index in models to predict chewing activity, ruminal retention, passage rate and digestibility. Fragility may be related to lignin content and digestibility as well as some of the anatomical differences such as cell wall thickness among forages.[7] According to Table 4 the fragility index was lower for wheat straw and orchard grass compared to alfalfa hay and corn silage, respectively. The greater NDFD of alfalfa hay versus wheat straw can be related to the greater susceptibility to particle size reduction.[11] This likely reflects the lower NDF and ADL contents for alfalfa hay as opposed to wheat straw.[11] Forage NDFD can be used successfully as a diagnostic tool to evaluate forage quality when NDF concentrations are similar, but it cannot be used directly in rations formulation.[23]

Figure 1 shows relationships between fragility and NDFD at 96, 120 and 240 hr, respectively. Grant and Zali *et al.* have observed that there is a positive relationship between NDFD and fragility, it appears that fragility may be more useful in adjusting peNDF values than digestibility.[2,11] Grant has concluded that NDFD and fragility are related and this relationship can be used to improve predictions of chewing response to peNDF.[11]

Because measurements of NDFD and fragility can be highly variable, it is possible that a ball milling method would have much less variation associated with it. In line with that, the relationship between NDFD and fragility is needed to be tight. More samples are needed to be analyzed to know the true relationship between NDFD and fragility, although at this point, we are assuming that the general relationships shown in 1 are true.

In conclusion, although lignin plays a central role in cell wall degradation and iNDF of plant materials, its concentration cannot be used for estimation of iNDF or pdNDF digestibility across a wide range of feed samples. Determination of uNDF should be included in routine forage and feed analysis because iNDF are uniform feed fractions with a predictable digestibility (i.e. 0). In contrast, NDF are non-uniform feed fractions containing multiple pools that digest predictably as a primary function of lignification. Further development of mechanistic models will be required for proper disclosing of diet composition effects on iNDF concentrations. Thus, the *in situ* incubation method can be considered as an invaluable tool in forage evaluating techniques in ruminant nutrition. The relationship of NDFD and fragility can be used to improve prediction of chewing response to peNDF. Assessment of forage physical properties can be used to precisely predict chewing and productive responses of dairy cattle.

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Conflict of Interest

The authors declare no conflict of interest.

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