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# Relationship between intake and faecal excretion of indigestible fractions in trials with sheep: impact of the method of analysis, diet and trial

C. A. Pozo[1,2], M. P. Mezzomo[1], J. Frasson[1], L. E. Leonardi[1], C. M. Christmann[1], D. C. Rösler[1] and G. V. Kozloski[1]

[1]Department of Animal Science, Federal University of Santa Maria, Santa Maria, 97105-900, RS, Brazil and [2]CIT-Formosa, CONICET, Faculty of Natural Resources, National University of Formosa, Formosa, 3600, Argentina

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**Author for correspondence:**
G. V. Kozloski,
E-mail: gilberto.kozloski@ufsm.br

## Abstract

A data set of individual observations was compiled from 17 digestibility trials with sheep with the aim of evaluating the impact of the method of analysis and, within each method, the effect of dietary fibre content and trial on the faecal recovery rate (RR) of indigestible dry matter (iDM) and indigestible neutral detergent fibre (iNDF). Markers concentration in samples of diet and faeces were determined either in polyester filter bags of 40 µm of porosity incubated *in situ* for 144 h (method A; $n = 257$) or in bags of 16 µm of porosity incubated *in situ* for 288 h (method B; $n = 321$). Regardless of the incubation method, the intake of either iDM or iNDF was linearly ($P < 0.05$) related to the amount excreted in faeces and, for all linear relationships, the slopes were lower than 1 (i.e. between 0.53 and 0.71). Within both methods, the RR of both markers linearly decreased at increased NDF content in diet ($P < 0.05$). The root mean square error of the linear relationships varied from 0.147 to 0.189 which represented from 19% to 24% of the respective average RR of markers. In conclusion, regardless of the incubation method, the RR of both markers showed high individual variability and, thus, the use of average RR values obtained in controlled digestibility trials as an index to correct the individual digestibility values obtained with either marker in field trials has the potential to improve the accuracy but not the precision of the digestibility estimates.

## Introduction

Feed intake and digestibility are the main factors impacting animal productive performance (Mertens, 1994). However, particularly in experiments with grazing ruminants, both dry matter (DM) intake and digestibility cannot be directly measured and indirect methods are used to estimate these variables. Diet digestibility can be estimated through *in vitro* or *in situ* techniques which, however, are under criticism because they do not simulate all animal digestion processes and, additionally, the *in vitro* assays are under high variability between measurement series (Peyraud, 1997). Alternatively, diet digestibility can also be estimated by using internal markers, which are unitary chemical entities of feedstuffs which are expected to be completely excreted in faeces (Lippke, 2002).

Either the indigestible dry matter (iDM) or the indigestible neutral detergent fibre (iNDF) is commonly used as an internal marker and, in general, their concentration is determined by incubating feed and faecal samples *in vitro* (Cochran *et al.*, 1986; Lippke *et al.*, 1986) or *in situ* (Tamminga *et al.*, 1989) for a long period of time and by assuming that the residual DM or neutral detergent fibre (NDF) are chemically identical in feed and faeces. However, although it is assumed that both markers have zero digestibility, the faecal recovery rate (RR) of iDM and iNDF reported in previous studies have been variable and different from 100% (Lippke *et al.*, 1986; Huhtanen *et al.*, 1994). Moreover, there is not a standard technique to determine these markers and previous studies have evaluated the impact of bag porosity and/or incubation time on iDM or iNDF concentration in feedstuffs or even their reliability on estimating the *in vivo* digestibility (Tamminga *et al.*, 1989; Huhtannen *et al.*, 1994; Berchielli *et al.*, 2000; Casali *et al.*, 2009; Krizsan and Huhtannen, 2013; Lee and Hristov, 2013; Krizsan *et al.*, 2015; Norris *et al.*, 2019). Results of these studies have been broadly variable and, in fact, the reliability of using a marker for estimating diet digestibility is greatly dependent on its RR in faeces. However, most published studies which have used either internal marker to estimate diet digestibility assumed that its faecal RR was 100% or that the average RR measured within the trial was the same in all experimental animals. However, it is relevant to consider that the individual variability on marker RR may impact the accuracy of individual digestibility estimates and, as a consequence, may produce a bias on diet treatment means in field trials.



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The aim of this study was to evaluate the impact of the *in situ* technique, NDF content in diet and trial on the relationship between intake and faecal excretion and, consequently, on faecal RR of iDM and iNDF in trials with sheep.

## Materials and methods

### *Data set and* in situ *techniques*

A data set of individual observations was compiled from digestibility trials conducted at the Federal University of Santa Maria, Santa Maria, RS, Brazil (S 29°29′, W 54°13′, a.l. 102 m) with sheep (17 trials) housed in metabolic cages and fed only forage or forage plus supplements. All trials were conducted using a Latin Square design with experimental periods varying from 15 to 21 days, with 10 to 14 days for adaptation and 5 to 7 days for measurements, when the feed offered, refusals and faeces were weighed, recorded, and sampled. General description of the experiments and relevant variables are presented in Table 1. All samples were dried at 55°C for at least 72 h, ground through a 1-mm screen and pooled by the animal within each experimental period for analysis. Total DM content was determined by oven drying at 105°C for 24 h. Samples of diet and faeces from 8 trials ($n = 257$) were weighed (2 g) in triplicate in polyester filter bags (5 × 5 cm, 40 µm of porosity) and incubated for 144 h in the rumen (method A) of a cannulated steer grazing tropical or temperate grass pastures and receiving supplementation with concentrate feedstuffs. This incubation time was defined based on results reported by Lippke *et al.* (1986) who observed that RR of indigestible fibre in faeces of sheep did not change appreciably after 144 h of *in vitro* incubation of feed and faeces samples. After rumen incubation, the bags were rinsed with tap water until the water remained clear, dried at 105°C for 24 h and weighed to obtain the iDM content. Thereafter, the bags containing the dried residues were treated with a neutral detergent solution in an autoclave at 110°C for 40 min (Senger *et al.*, 2008), washed in tap water, dried at 105°C for 24 h and weighed to obtain the iNDF content. Alternatively, samples of diet and faeces of 13 trials ($n = 321$) were also incubated and treated as described above, except that they were weighed in bags (5 × 5 cm) with 16 µm of porosity and incubated *in situ* for 288 h (method B), as proposed by Krizsan and Huhtannen (2013). The daily intake and faecal excretion (g/kg body weight (BW)) of both iDM and iNDF were calculated as: DM intake or faecal output (g/kg BW) × marker concentration (g/g DM). Marker RR was calculated as: marker excreted in faeces (g/kg BW)/marker intake (g/kg BW).

### *Statistical analysis*

In two trials some experimental treatments included tannins and, once tannins complex with carbohydrates and proteins in the digestive tract producing neutral detergent insoluble compounds which are excreted in faeces (Van Soest, 1994), data of animals receiving tannins were excluded from the analysis. Samples of only four trials were used to test both methods and thus, results from both methods were not directly compared. The linear relationship between ingestion and faecal excretion of either marker within each method was performed using the PROC MIXED of SAS (SAS Institute Inc., Cary, NC, USA), including the random effect of trial in the model. The confidence interval (95%) of the equation parameters were calculated on the basis of standard error (SE) values (i.e. ± 2 S.E.) and used to evaluate the deviation of either the slope from 1 or intercept from 0. Significance was declared at $P \leq 0.05$. The effect of trials on the average RR of markers within each method was analysed using the PROC GLM of SAS. The standard deviation of means within trials was assumed to indicate the individual variability on RR values within trials. The potential impact of the NDF content in diet on individual RR of markers within each method was determined through linear relationship using the PROC MIXED of SAS, including the random effect of trial in the model.

## Results

The intake of either iDM or iNDF determined by method A was linearly ($P < 0.05$) related to the amount excreted in faeces (Fig. 1). The intercept of both regressions was not different from 0 whereas the slopes were lower than 1 (i.e. 0.71 and 0.53 for iDM and iNDF, respectively, $P < 0.05$). The RMSE of the linear relationship was 0.745 for iDM and 0.225 for iNDF. The average RR was different between trials ($P < 0.05$) with values varying from 0.64 to 1.08 for iDM and from 0.49 to 0.92 for iNDF (Fig. 2). The standard deviation within trials varied from 9% to 30% of their respective average RR values for iDM, and from 10% to 38% for iNDF.

As by method A, the intake of either marker determined by method B was also linearly ($P < 0.05$) related to the amount excreted in faeces (Fig. 3). In both linear regressions the intercept was higher than 0 ($P < 0.05$) and the slope was lower than 1 (i.e. 0.70 and 0.69 for iDM and iNDF, respectively, $P < 0.05$). The RMSE of these linear relationships were 1.095 for iDM and 0.880 for iNDF, respectively. The average RR was also different between trials ($P < 0.05$) with values varying from 0.61 to 1.32 for iDM and from 0.57 to 1.23 for iNDF (Fig. 4). The standard deviation within trials varied from 5% to 36% of their respective average RR values for iDM, and from 5% to 38% for iNDF.

Within both methods, the RR of both markers linearly decreased at increased NDF content in diet ($P < 0.05$, Fig. 5). The RMSE of the linear relationships varied from 0.147 to 0.189 which represented from 19% to 24% of the respective average RR of markers. The general RR of iDM and iNDF were on average 0.90 and 0.61 by Method A and 1.00 and 0.97 by Method B, respectively.

## Discussion

Several published digestibility studies, as Berchielli *et al.* (2000), Berchielli *et al.* (2005) and Casali *et al.* (2009), have determined the indigestible fractions of DM or NDF by weighing the samples in bags with 40 to 50 µm of porosity and incubating them *in situ* or *in vitro* for 144 h. However, this methodology was under criticism due to a potential loss of undegraded sample through the bag pores and/or incomplete and variable degradation of the indigestible fraction at this relatively short incubation time (Huhtanen *et al.*, 1994; Krizsan and Huhtanen, 2013; Krizsan *et al.*, 2015). As to obtain more biologically accurate estimates of the NDF fraction that would be unavailable for microbial digestion in ruminants, an alternative technique was initially proposed by Huhtanen *et al.* (1994) and further evaluated by Krizsan and Huhtanen (2013), by which feed and faecal samples are incubated *in situ* during 288 h in bags with very low porosity (i.e. 6 or 12 µm). In fact, in the present study, when calculated as the simple mean value of the individual RR values, the RR of both markers was on average higher and closer to 100% by Method B than by

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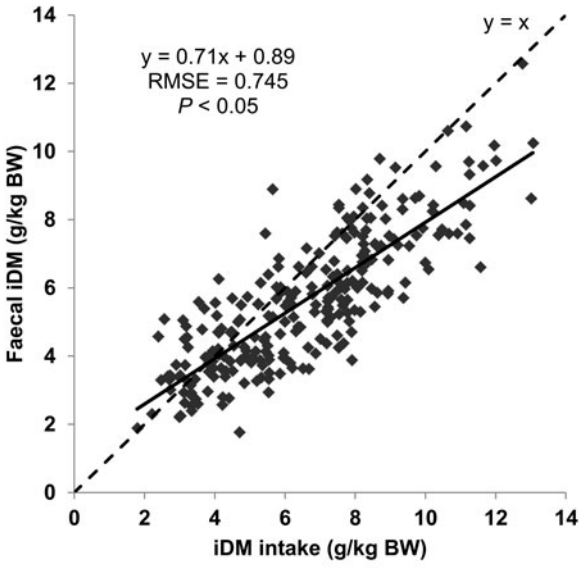
**Table 1.** Descriptive variables of digestibility trials carried out with sheep between 2003 and 2018 at the Universidade Federal de Santa Maria, Santa Maria, RS, Brazil (29°4′S, 53°5′W)

| | | | | | Intake, g/d | | | | Faecal output, g/d | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Trial[a] | *n* | Diet | Method[b] | BW (kg) | DM | NDF | iDM | iNDF | DM | iDM | iNDF |
| 1 | 18 | *Cynodon dactylon* hay at levels of intake | A | 28–38 | 373–1055 | 267–754 | 72–202 | 52–147 | 193–429 | 73–186 | 42–130 |
| 2 | 18 | Native grassland hay at levels of intake | A | 28–38 | 177–632 | 133–492 | 67–240 | 52–184 | 137–445 | 70–191 | 38–130 |
| 3 | 45 | *Lolium multiflorum* fresh alone or plus either sodium caseinate, cassava meal or corn gluten meal | A | 22–30 | 499–1182 | 165–539 | 44–191 | 34–136 | 110–305 | 46–160 | 20–84 |
| 4 | 17 | *Pennisetum clandestinum* hay at different regrowth ages | A | 32–38 | 606–1069 | 406–698 | 167–294 | 103–181 | 105–441 | 63–245 | 31–103 |
| 5 | A: 31; B: 32 | *Pennisetum clandestinum* hay plus cassava meal, calcium caseinate and/or urea | A; B | 29–45 | 517–1184 | 242–653 | A:124–367; B: 85–235 | A: 95–282; B: 73–201 | 212–507 | A:106–292; B: 103–261 | A: 41–167; B: 83–204 |
| 6 | 50 | *Cynodon dactylon* hay alone or plus urea, cassava meal and/or calcium caseinate | A;B | 15–30 | 321–814 | 141–572 | A: 65–301; B: 51–227 | A: 50–231; B: 45–201 | 104–342 | A: 50–257; B: 47–204 | A: 25–113; B: 41–176 |
| 7 | A: 28; B: 29 | *Echinochloa sp.* hay at different regrowth ages | A; B | 27–38 | 533–1265 | 405–899 | A:175–357; B: 161–347 | A: 113–260; B: 138–296 | 213–607 | A: 144–344;B: 135–350 | A: 59–166;B: 109–295 |
| 8 | 50 | *Cynodon dactylon* hay alone or plus urea and levels of cassava meal | A; B | 17–50 | 437–1118 | 215–692 | A: 92–361; B: 161–347 | A: 60–236; B: 138–296 | 153–271 | A: 102–319; B: 82–285 | A: 46–156; B: 73–250 |
| 9 | 17 | Native grassland hay at levels of intake | B | 15–27 | 224–413 | 185–341 | 72–157 | 64–139 | 142–251 | 102–189 | 82–151 |
| 10 | 5 | *Cynodon dactylon* hay plus corn gluten feed | B | 37–46 | 622–835 | 382–543 | 140–234 | 123–209 | 230–300 | 157–225 | 135–195 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 11 | 31 | Corn silage plus soybean meal, corn cracked grain and/or sweet potato flour | B | 15–35 | 462–1141 | 136–453 | 60–137 | 48–114 | 54–345 | 23–166 | 20–140 |
| 12 | 32 | *Avena strigosa* hay plus levels of sweet potato flour and urea | B | 28–46 | 541–1313 | 251–637 | 69–358 | 66–320 | 194–439 | 143–369 | 121–319 |
| 13 | 32 | *Sorghum sudanense* plus corn cracked grain and levels of canola meal | B | 26–35 | 331–963 | 216–558 | 107–301 | 92–260 | 76–345 | 46–262 | 35–213 |
| 14 | 16 | *Lolium multiflorum* fresh at levels of intake | B | 25–28 | 369–1047 | 229–641 | 115–328 | 98–278 | 174–379 | 118–273 | 93–225 |
| 15 | 16 | *Cynodon dactylon* fresh at levels of intake | B | 23–31 | 315–849 | 237–616 | 103–278 | 87–234 | 106–459 | 79–328 | 65–286 |
| 16 | 6 | *Cynodon dactylon* hay plus soybean meal, wheat bran, corn cracked grain | B | 56–78 | 1656–1960 | 921–1158 | 440–572 | 400–518 | 385–587 | 241–353 | 205–303 |
| 17 | 5 | *Avena strigosa*/ *Lolium multiflorum* mixed hay plus soybean meal, rice bran, corn cracked grain | B | 47–56 | 1431–1673 | 715–914 | 399–506 | 327–425 | 572–750 | 352–466 | 293–394 |

[a]References: Trials 1, 2, 9,12, 14 and 15, unpublished; Trial 3, Amaral *et al.* (2011); Trial 4, Ruggia Chiesa *et al.* (2008); Trial 5, Kozloski *et al.* (2009*b*); Trial 6, Kozloski *et al.* (2007); Trial 7, Lima *et al.* (2008); Trial 8, Kozloski *et al.* (2006); Trial 10, Stefanello *et al.* (2018); Trial 11, Mibach *et al.* (2021); Trial 13, Hentz *et al.* (2012); Trial 16, Orlandi *et al.* (2020*a*); Trial 17, Orlandi *et al.* (2020*b*).
[b]Method A: 144 h of *in situ* incubation of samples in bags of 40 μm porosity; Method B: 288 h of *in situ* incubation of samples in bags of 16 μm porosity.



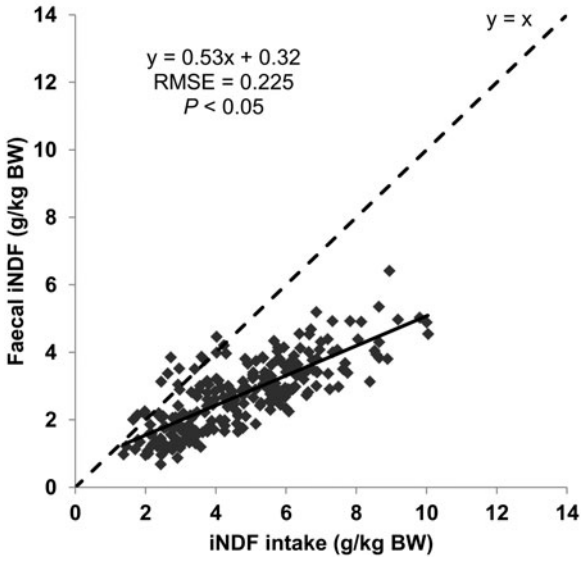




**Fig. 1.** Relationship between intake and faecal output of indigestible dry matter (iDM) or indigestible neutral detergent fibre (iNDF) in trials with sheep (8 trials, $n = 257$). Description of trials is shown in Table 1. Total faeces were collected during the last 5 days of experimental periods varying from 14 to 17 days. Indigestible fractions were obtained after 144 h of *in situ* incubation of feed and faecal samples in bags of 40 μm porosity. For both linear relationships, the intercept was not different from 0 and the slope was different from 1 ($P < 0.05$). BW, body weight; RMSE, root mean square error.

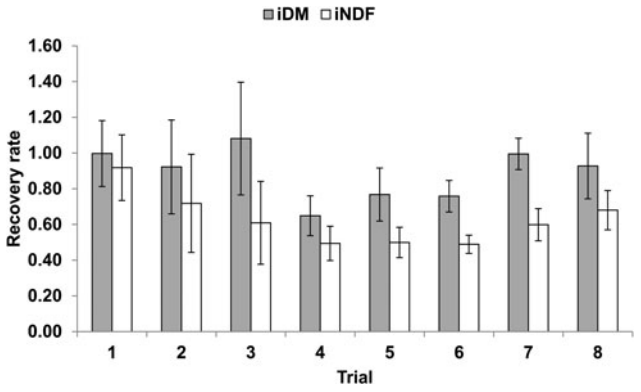



**Fig. 2.** Mean recovery rates of indigestible dry matter (iDM) and indigestible neutral detergent fibre (iNDF) in trials with sheep. Description of trials is shown in Table 1. Total faeces were collected during the last 5 days of experimental periods varying from 14 to 17 days. Indigestible fractions were obtained after 144 h of *in situ* incubation of feed and faecal samples in bags of 40 μm porosity. Bars on columns are standard deviation of means. Effect of trial: $P < 0.05$.

Method A. However, by assuming the slope of the linear regressions between intake and faecal excretion as an index of markers RR (i.e. a slope of 1.0 meaning 100% of RR), both methods were equally inaccurate (i.e. how much the slope was different from 1) on determining iDM whereas the performance on determining iNDF was lower by Method A than by Method B (i.e. slope of 0.53 *v.* 0.69, respectively).

Regardless of the method of marker determination, there was a high variability on RR between trials with some of them showing average values below and others above 100%, which is in accordance with results previously reported by Kozloski *et al.* (2009*a*) and other groups. For example, the RR of iNDF determined *in vitro* and reported by Lippke *et al.* (1986) in trials with sheep and cattle varied from an average 83% to 130% whereas Huhtanen *et al.* (1994) obtained average RR for iDM and iNDF determined *in situ* varying from 83% to 116% in trials with cattle. In the present study, the differences on RR between trials could be due, at least partially, to differences on diet types. Velazquez *et al.* (2021), for example, reported increased RR of iNDF in trials with Nelore bulls fed grass hay compared to those fed corn silage based diets and, in the study of Krizsan and Huhtanen (2013), the iNDF content in feed samples incubated *in situ* increased at an increased proportion of concentrate in diet of rumen fistulated cows. Moreover, in dairy cows fed diet with low but not in those fed diet with adequate crude protein (CP) content, the faecal output was overestimated and digestibility underestimated when iNDF was used as a marker (Lee and Hristov, 2013). In the present study, the CP content of diets was not broadly variable across the trials (data not shown) and, thus, only the potential impact of the NDF content in diet on markers RR was analysed. Regardless of the method of determination, the RR of both markers linearly decreased at increased NDF content of sheep diets. However, these linear relationships were not consistent showing a high individual variability (i.e. high RMSE values). A clear explanation for the NDF effect, and its variability, on RR is not available. However, it could be linked to the grinding process of feeds and faeces samples. Even though all feed and faecal samples were grounded to pass a 1 mm screen in the present study, faecal samples consist of a more fragmented and uniform material, containing fibre particles which are probably not more susceptible to further mechanical breakdown during the grinding process. In turn, the grinding process of forage samples, mainly of those of low quality, usually results in a more heterogeneous material containing since very small powder particles to particles longer than the 1 mm sieve screen. As a consequence, the susceptibility of this heterogeneous substrate to microbial degradation during the *in situ* incubation could be also variable yielding variable contents of iNDF (Ovani *et al.*, 2022) even with at incubation time as long as 288 h. Recently, Norris *et al.* (2019) and Adams *et al.* (2020) reported that the iDM and iNDF fractions in feeds and faeces decreased by increasing the time of *in situ* incubation from 288 to 576 h. However, the impact of the longer incubation time was more pronounced in feed than in faeces samples.

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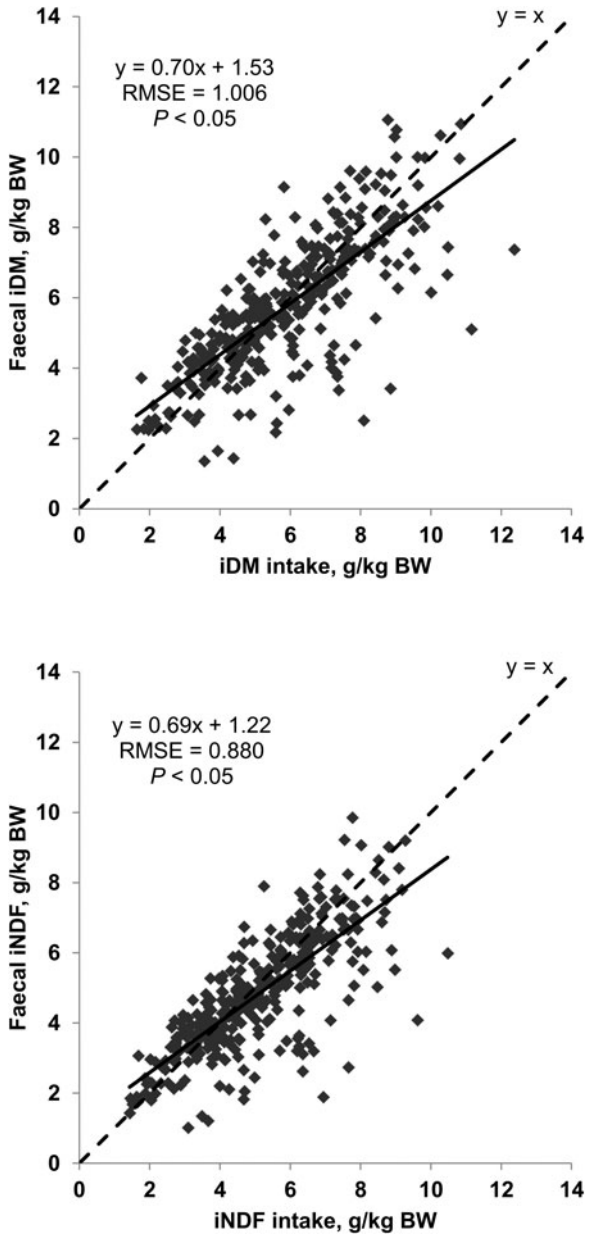


**Fig. 3.** Relationship between intake and faecal output of indigestible dry matter (iDM) or indigestible neutral detergent fibre (iNDF) in trials with sheep (13 trials, $n = 321$). Description of trials is shown in Table 1. Total faeces were collected during the last 5 days of experimental periods varying from 14 to 17 days. Indigestible fractions were obtained after 288 h of *in situ* incubation of feed and faecal samples in bags of 16 μm porosity. For both relationships the intercept and the slope of the linear regressions were different from 0 and 1, respectively ($P < 0.05$). BW, body weight; RMSE, root mean square error.

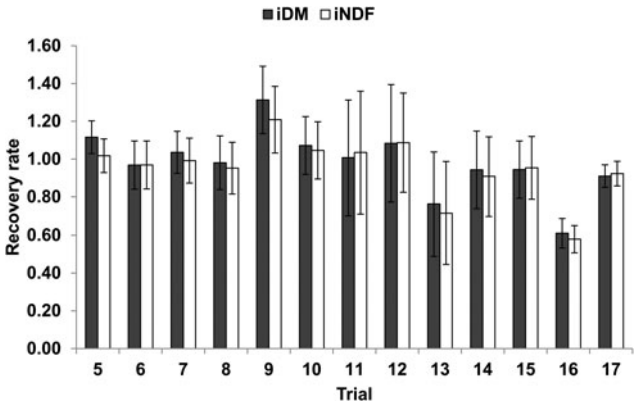


**Fig. 4.** Mean recovery rates of indigestible dry matter (iDM) and indigestible neutral detergent fibre (iNDF) in trials with sheep. Description of trials is shown in Table 1. Total faeces were collected during the last 5 days of experimental periods varying from 14 to 17 days. Indigestible fractions were obtained after 288 h of *in situ* incubation of feed and faecal samples in bags of 16 μm porosity. Bars on columns are standard deviation of means. Effect of trial: $P < 0.05$.

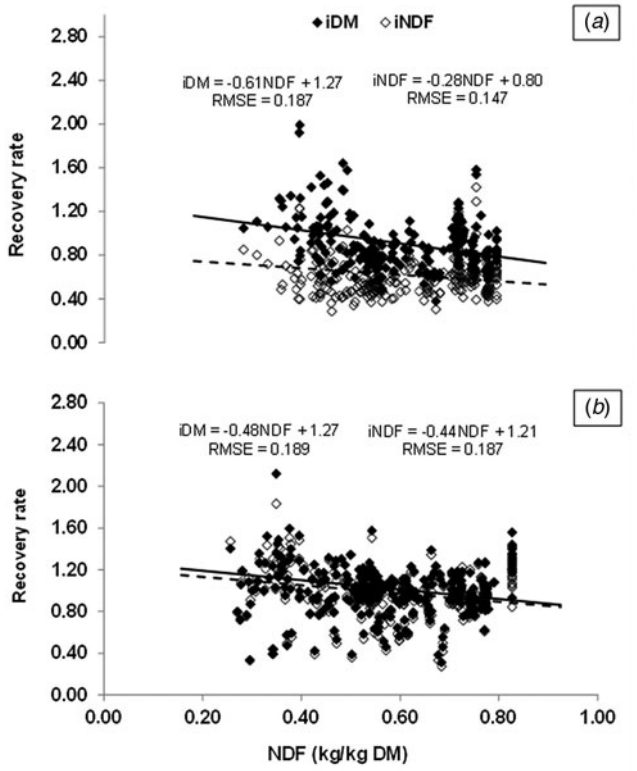


**Fig. 5.** Relationship between neutral detergent fibre (NDF) content in diet and faecal recovery rate of indigestible dry matter (iDM) and indigestible neutral detergent fibre (iNDF) in trials with sheep. Description of trials is shown in Table 1. Total faeces were collected during the last 5 days of experimental periods varying from 14 to 17 days. Indigestible fractions were obtained after 144 h of *in situ* incubation of feed and faecal samples in bags of 40 μm porosity (A, $n = 257$) or after 288 h of *in situ* incubation of feed and faecal samples in bags of 16 μm porosity (B, $n = 321$). RMSE, root mean square error. Effect of NDF content in diet for each marker within method: $P < 0.05$.

On average of both studies, whereas the iDM and iNDF contents in feed samples decreased by approximately 21% this reduction was of only 3% in faeces samples.

As relevant as the variability observed between trials, there was also a high variability of markers RR between animals within trials, which is indicated by both the relatively high RMSE values of the linear relationships between intake and faecal excretion of markers across all trials and the high standard deviation of the mean values within trials. A high individual variability on RR values of iNDF was also reported by Berchielli *et al.* (2005) in trials with beef cattle. As a consequence, it would be expected that individual digestibility values estimated with either marker in a field trial would be under bias, regardless they are corrected or not for a mean RR obtained in a controlled digestibility trial. Thus, even though the accuracy of markers RR was apparently improved, not relevant precision (i.e. how much the RMSE or

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standard deviation value is close to 0) advantage was observed in the present study by using bags with lower porosity and longer *in situ* incubation time for determining iDM or iNDF.

## Conclusion

Regardless of the incubation method, the faecal RR of both iDM and iNDF obtained in digestibility trials with sheep showed high variability not only between trials but also within trials. As a consequence, using average RR values obtained in controlled digestibility trials as an index to correct the individual digestibility values obtained with either marker in field trials have the potential to improve the accuracy but not the precision of the digestibility estimates.

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**Author contributions.** G. V. Kozloski and C. A. Pozo conceived and designed the study; C. A. Pozo, M. P. Mezzomo, J. Frasson, L. E. Leonardi, C. M. Christmann and D. C. Rösler conducted data gathering; G. V. Kozloski and C. A. Pozo performed statistical analyses; G. V. Kozloski and C. A. Pozo wrote the article.

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**Conflict of interest.** The authors declare there are no conflicts of interest.

**Ethical standards.** All experimental procedures in trials contributing to the present study complied with the regulations governing the use of animals in the experimentation of the Federal University of Santa Maria, Santa Maria, RS, Brazil.

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