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an integrated infrastructure for increased research capability and innovation in the European cattle sector

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EXECUTIVE SUMMARY

Background	Research activities in SmartCow (https://www.smartcow.eu/) aimed to increase phenotyping capabilities while implementing the 3R principles (refine, reduce and replace) in cattle nutrition and behaviour studies. Development and validation of non-invasive proxies of feed efficiency (FE) and its determinants were undertaken with the goal of minimizing handling and constraints of experimental cattle (WP6) in research infrastructures (RIs). Proxies are defined as "indicators" measurable in cattle body matrices easy to access and easier to implement than the gold standard methods (GSM) used to measure the phenotype of interest.
Objectives	The objectives of this work were: **1) to assess the potential and limits of different proxies to predict feed efficiency and its determinants in cattle across diets and individuals**. **We focused on the most promising proxies for their practical application at large scale on farm.** This concern: - the natural ^{15}N abundance in animal proteins for prediction of feed efficiency (FE) in beef cattle (plasma) and milk nitrogen use efficiency (MNE) in dairy cows (milk); - near-infrared spectra (NIRS) in faeces for prediction of total-tract digestibility (OMD); milk mid-infrared spectra (MIRS) for predicting CH_4 emissions in dairy cows. The potential of NIRS in faeces to predict CH_4 emissions in cattle **2) to edit recommendations for RIs and stakeholders (academic, industrial) on the use of the proxies according to common and standardized protocols.** For each proxy, the principle by which it is related to the phenotype, the model of prediction with its domain of validity and its accuracy, and its advantages and drawbacks are presented as guidelines form.
Methods	Our strategy consisted in building a large and representative database of the European breeding conditions (as far as possible) including both individual phenotypes (FE, MNE, OMD, CH_4) measured using reference methods, and proxies (15N, NIRS, MIRS) from different easily accessible body matrices (milk, faeces, blood) from beef and dairy cattle. This database building was possible thanks to a strong collaborative network among SmartCow partners but also with collaborators outside the consortium (TNA applicants including private companies and other research institutes like LUKE from Finland and Agroscope from Switzerland). Collection of data and proxies were carried out from historical experiments and new experiments conducted during the SmartCow project. When proxies were not available, samples were transported to the laboratory for analyses according to standardized sampling protocols. Models were tested for different proxies to predict phenotypes across diets and between-individuals.
Results & implications	**^{15}N for prediction of FE in beef cattle and MNE in dairy cows** Meta-analysis demonstrated that the natural ^{15}N abundance in animal proteins has a stronger predictive ability than plasma urea to discriminate dietary treatments, as well as individual variation in FE of beef cattle and MNE of dairy cattle. For more details, see publications https://doi.org/10.5281/zenodo.6500307 and https://doi.org/10.3168/jds.2021-21498.

Models are published as open data and readily available by users to predict FE or MNE from their own ^{15}N data analysis.

Faecal NIRS for predicting OMD and enteric CH_4 emissions in cattle

Models based on faecal near-infrared spectra (NIRS) discriminated dietary treatments and extreme individuals in terms of OMD in dairy and beef cattle, with an error of prediction close to that of the GSM (6.4% vs 5.2%, respectively). Data confirm the good potential of faecal NIRS as a proxy for OMD prediction in cattle. Publication of these models are in progress. The standardized models are available for users (contact: donato.andueza@inrae.fr).

First results on the potential of faecal NIRS to predict enteric CH_4 emissions are promising. This innovative proxy represents a great practical interest in non-lactating animals. More CH_4 reference data and corresponding faecal NIRS are required to confirm the trends observed on beef cattle, improve the robustness of this proxy and enlarge the range of application of the model (other breeds, diets, physiological status). Contact donato.andueza@inrae.fr if you have data of interest (OMD, CH_4) and faecal samples to share.

Milk MIRS for predicting enteric CH_4 emissions in dairy cows

For dairy cows, milk mid-infrared spectra (MIRS) existing model predicted CH_4 emissions with an error of prediction of 58 g/d. This high throughput approach (low cost and easily available analysis performed in milk recording laboratory) offers the possibility to integrate CH_4 emissions in dairy cow selection programs. The existing model based on SF_6 and respiratory chamber reference data is currently implemented in different countries and could be accessed through the web service proposed by EMR (spectral standardization + prediction; contact jleblois@awegroupe.be). In addition, a new equation of prediction using CH_4 reference data measured with reference values obtained with the GreenFeed system is in progress. Improving the robustness of the models requires new reference data not yet represented in the database. Contact a.vanlierde@cra.wallonie to collaborate if you have data of interest to share.

We observed that for some phenotypes (OMD, CH_4), it is not possible to merge reference data sets measured with different methods without including noise in the models of prediction. **This highlights the importance of common and standardized protocols for measurements, sampling and data recording before merging and enhancing all future data. This is an essential step to enlarge the diversity of the reference database with data of quality and to update the models according to research recommendations.**

Guidelines on the use of the proxies proposed in this document will be published in the book of method of SmartCow as open access guidelines for RIs and stakeholders (academic and industry)
https://books.publisso.de/en/publisso_gold/publishing/books/overview/53/186/about**.**
This would help phenotyping capacity of the RIs. In addition, adoption and implementation of these proxies should constitute an interesting phenotyping tools for feed and breeding industry for enhancing competitiveness and sustainability of the livestock sector.

Table of contents

1 Natural ^{15}N abundance as a biomarker of feed efficiency in cattle

1.1 Principle by which the biomarker is related to the phenotype

Nitrogen (N) naturally exists as two stable isotopes, the ^{14}N which is the lightest form and the far more abundant in the nature (on average 99.636%) and the less abundant heavy ^{15}N (on average 0.364%). **Atomic mass differences between these two isotopes (14.006 vs 15.0001 u) explains why they behave slightly different in a variety of biochemical reactions**. In particular, the energy needed to cleave molecular bonds during some biochemical reactions is lower when ^{14}N is involved compared to ^{15}N, leading to reactions that run slightly faster when compounds contain the lightest vs heaviest isotope (i.e. *isotope effect*). Indeed, some studies reported that **enzymatic reactions dealing with nitrogenous compounds react faster with (or have greater affinity by) ^{14}N vs ^{15}N containing compounds (Macko et al. 1986; Yoneyama et al., 1993)**. This leads to a different isotopic composition between substrates and products, phenomenon known as *isotopic fractionation*. **Enzymatic reactions favoring ^{14}N vs ^{15}N at the molecular level has a measurable impact at the organism level: ^{15}N natural abundance ($\delta^{15}N$) in animal proteins is usually higher than in the diet consumed (DeNiro and Epstein, 1981), phenomenon known as *N isotopic discrimination* ($\Delta^{15}N_{animal\text{-}diet}$).**

In ruminants, results from few studies suggest that main fractionating process for N isotopes are those related with the balance between ammonia uptake and release by rumen bacteria **(Wattiaux and Reed, 1995; Cantalapiedra-Hijar et al., 2016)** and the transamination pathway involved in hepatic amino acids catabolism (Cantalapiedra-Hijar et al., 2015). These two phenomena are strongly involved in the overall efficiency of N utilization by the ruminants as well **(INRA, 2018)** and therefore are thought to be responsible for the link between $\Delta^{15}N_{animal\text{-}diet}$ and the ability of ruminant to assimilate feed N **(Cantalapiedra-Hijar et al., 2018)**. **When the ratio of ammonia uptake/release by rumen bacteria is improved and the liver amino acid catabolism decreases, the overall efficiency of N utilization increases at the same time that $\Delta^{15}N$ decreases (Cantalapiedra-Hijar et al., 2016)**. The natural ^{15}N abundances represent thus a real metabolic signature on the way the organisms partition the N between anabolism and catabolism and have been shown to reflect the efficiency of N assimilation in different livestock species **(Gaye-Siessegger et al., 2004; Sears et al., 2009; Cheng et al., 2013)**, plants **(Fuertes-Mendizabal et al., 2018)** and humans **(Fuller et al., 2004)**.

Two previous meta-analysis highlighted that $\Delta^{15}N$ reflects the N use efficiency in dairy cows (Cantalapiedra-Hijar et al., 2018) and feed efficiency in beef cattle **(Guarnido Lopez et al., 2021)** and maybe useful for predicting both phenotypes across diets but between-individuals. However, previous meta-analysis studies in ruminants were conducted with a small database or in specific conditions in terms of breed and diets. The work conducted during the Smartcow project allowed to better define the potential and drawbacks of this new biomarker of nutrient use efficiency in ruminants.

1.2 Prediction equations

Feed efficiency metrics investigated were: **Feed Conversion Efficiency** (**FCE**, kg/kg) measured as the average daily gain (kg/d) divided by the DM intake (kg/d), Residual Feed Intake (**RFI**, kg/d) calculated as the observed DM intake (kg/d) minus the expected DM intake (kg/d), the latter estimated at the contemporary group level from metabolic body weight and average daily gain, and milk N use efficiency (**MNE**, g/g) calculated as the N output in milk (g/d) divided by N intake (g/d)

GSM method for FCE and RFI consisted in recording during at least 56 days the inidivudal and daily DM intake as well as individual body weight every either 1 or 2 or 4 weeks.
GSM for MNE determination consisted in recording during at least 4 days the individual and daily N intake as well as the milk N (or alternatively true protein) output.

1.2.1 Feed efficiency in beef cattle (FCE, RFI)

1.2.1.1 Comparing FCE across diets

- **Equation:** FCE (kg/kg) = 0.273 – 0.032 × $\Delta^{15}N_{animal\text{-}diet}$ (RMSE = 0.018 kg/kg; n = 749; n_diets = 34; r = 0.56; P<0.001)

- **Domain of validity:** growing and fattening beef cattle (young bulls, steers and heifers) fed fattening diets with a high proportion of forages (>50%) and with FCE ranging between 0.03 and 0.29 kg/kg. Breeds tested concerned mainly Charolais, Limousine, Simmental and crossed-bred. Description of experimental conditions used for developing prediction equations can be found here: https://**doi.org/10.5281/zenodo.5771504**
- **Precision:** The minimum detectable difference in FCE with the proposed equation is 0.07 kg/kg (IC 95%) meaning that analyzing $\Delta^{15}N$ in two different animals randomly selected from the same farm will allow to discriminate their dietary treatment if they differ by at least 0.07 kg/kg. This minimum detectable difference in FCE would logically decrease as the number of animals per dietary treatment increase.
- **Example of power analysis:** With the obtained prediction error (0.018 kg/kg), we can discriminate (IC 95%) within the same farm, two diets promoting FCE differences of at least 0.02 kg/kg if $\Delta^{15}N$ is measured in 13 animals per diet.

1.2.1.2 Comparing FCE across individuals within the same contemporary group

- **Equation:** $FCE_{individual} - FCE_{mean}$ (kg/kg) = $-0.033 \times (\Delta^{15}N_{individual} - \Delta^{15}N_{mean})$ (RMSE = 0.016 kg/kg; n = 749; r = 0.57; P<0.001)

The equations should be read as follows: the difference between the FCE of any individual and the mean FCE of its respective contemporary group equals -0.033 times the difference between Δ15N of that particular individual and the mean Δ15N of its respective contemporary group. Mean could be replaced by another individual when comparing two specific animals. Contemporary group is defined as animals from the same study or farm, fed the same diet at the same time. For late maturing breeds (Charolais, Limousin, etc.) the slope should be -0.038 rather than -0.033 (P<0.05).

- **Domain of validity:** growing and fattening beef cattle (young bulls, steers and heifers) from the same contemporary group (same place, same diet and at the same time) fed fattening diets with a high proportion of forages (>50%) and with FCE ranging between 0.03 and 0.29 kg/kg. Breeds tested concerned mainly Charolais, Limousine, Simmental and crossed-bred. Description of experimental conditions used for developing prediction equations can be found here: https://**doi.org/10.5281/zenodo.5771504**
- **Precision:** The minimum detectable difference in FCE with the proposed equation is 0.06 kg/kg (IC 95%) meaning that $\Delta^{15}N$ is able to discriminate two animals from the same contemporary group if they differ by at least 0.06 kg/kg FCE. Alternatively, this biomarker can help to form groups of animals with similar feed efficiency (FCE) within a farm.
- **Example of power analysis:** With the obtained prediction error, we can theoretically detect a significant FCE difference of only 0.02 kg/kg (IC 95%) between two groups of 10 extreme feed efficiency cattle each by comparing their $\Delta^{15}N$ values (i.e. 10 efficient animals with FCE higher than 0.18 kg/kg vs 10 inefficient animals with FCE lower than 0.16 kg/kg).

1.2.1.3 Comparing RFI across individuals within the same contemporary group

Although $\Delta^{15}N$ has been shown to be significantly related to RFI in beef cattle (Guarnido Lopez et al., 2021; Cantalapiedra-Hijar et al., 2022) it cannot be proposed as a phenotyping tool to accurately discriminate two individuals based on their RFI (minimum detectable difference close to 1.5kg/d; Guarnido Lopez et al., 2021). However, $\Delta^{15}N$ may be a useful biomarker for forming groups of extreme animals in terms of RFI (this should be confirmed with further studies).

- **Example of power analysis:** With the obtained prediction error (0.20 kg/d), we can theoretically detect a significant RFI difference of 0.50 kg/d (IC 95%) between two groups of 25 extreme feed efficiency cattle each by comparing their $\Delta^{15}N$ values (i.e. 25 efficient animals with RFI values lower than -0.25 kg/d vs 25 inefficient animals with RFI higher than +0.25 kg/d).

1.2.2 Milk N use efficiency (MNE) in lactating dairy cows

1.2.2.1 Comparing MNE across diets

- **Equation:** MNE (g/g) = $0.407 - 0.050 \times \Delta^{15}N_{animal\text{-}diet}$ (RMSEP = 0.035 g/g; n = 1135; r = 0.57; P<0.001)

- **Domain of validity:** Lactating dairy cows (primiparous and multiparous) with days in milk higher than 50 days, with MNE ranging from 0.04 to 0.47 and fed diets with CP ranging between 11% and 27% (on DM basis) and concentrate between 8% to 82%. Most representative breed in the database was Holstein Friesian cows. Description of experimental conditions used for developing prediction equations can be found here: https://doi.org/10.3168/jds.2021-21498.
- **Precision:** The minimum detectable difference in MNE with the proposed equation is 0.14 g/g (IC 95%) meaning that analyzing $\Delta^{15}N$ in two different animals will allow to discriminate their dietary treatment if they differ by at least 0.14 g/g. This minimum detectable difference in MNE would logically decrease as the number of animals per dietary treatment increase.
- **Example of power analysis:** With the obtained prediction error we can theoretically discriminate (IC 95%) within the same farm two dietary treatments differing by at least 0.04 g/g of MNE if $\Delta^{15}N$ is measured in 24 extreme MNE dairy cows (i.e. 12 dairy cows fed a diet promoting MNE values higher than 0.30 g/g vs 12 dairy cows fed a diet promoting MNE values lower than 0.24 g/g).

1.2.2.2 Comparing MNE across individuals within the same contemporary group

- **Equation:** $MNE_{individual} - MNE_{mean}$ (g/g) = $- 0.056 \times (\Delta^{15}N_{individual} - \Delta^{15}N_{mean})$ (RMSEP = 0.028g/g; n = 1135; r = 0.60; P<0.001)

The equations should be read as follows: the difference between the MNE of any dairy cow and the mean MNE of its respective contemporary group equals -0.056 times the difference between $\Delta^{15}N$ of that particular cow and the mean $\Delta^{15}N$ of its respective contemporary group. *Mean could be replaced by another dairy cow when comparing two specific animals. Contemporary group is defined as those animals from the same study or farm, fed the same diet at the same time.*

- **Domain of validity:** Lactating dairy cows (primiparous and multiparous) with day in milk higher than 50 days and from the same contemporary group (same place, same diet and at the same time). MNE values ranging from 0.04 to 0.47 and diets with CP between 11% and 27% CP and concentrate ranging from 8% to 82%. Most representative breed in the database was Holstein Friesian cows. Description of experimental conditions used for developing prediction equations can be found here: https://doi.org/10.3168/jds.2021-21498
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- **Precision:** The minimum detectable difference in MNE with the proposed equation is 0.11 g/g (IC 95%) meaning that analyzing $\Delta^{15}N$ in two different animals from the same contemporary group will allow to discriminate their MNE if they differ by at least 0.11 g/g. This minimum detectable difference in MNE seems still too high for assisting breeding programs. Alternatively, this biomarker can help to form groups of dairy cows with similar MNE (high vs low) within a farm.
- **Example of power analysis:** With the obtained prediction error we can theoretically discriminate (IC 95%) within a farm two groups of dairy cows fed the same diet at the same time and differing by at least 0.03 kg/kg MNE by comparing the $\Delta^{15}N$ values of 28 extreme animals (i.e. 14 efficient animals with MNE higher than 0.30 g/g vs 14 inefficient animals with MNE lower than 0.27 g/g).

1.3 What is needed for using $\Delta^{15}N$ as a biomarker of feed efficiency?

1.3.1 Samples

- **If the goal is to compare animals in terms of feed efficiency for phenotyping purposes:** what is needed is a **biological sample containing animal proteins (plasma or milk** tested in the project; but hair might also be possible [Meale et al., 2017]) and obtained after at least 4 weeks after the introduction of a new diet (see limits of the biomarker further). The quantity of sample is relatively small (around 50µL of plasma or milk for having enough for analysis in duplicate) and the plasma should be obtained from blood with heparin as anticoagulant sampled from any blood vessel (usually caudal vein/artery or jugular vein) at any moment before or after meal distribution.

- **If the goal is to compare production contexts or dietary treatments rather than individuals:** it is also needed in addition **a representative sample of the whole diet or diet feed ingredients** that animals

received during the period of time being tested. The quantity of the representative feed samples is small and depends on the N content of feeds, with around 1-4 mg for concentrate feed and 3-9 for forages.

1.3.2 Analyses

Samples are analyzed by elemental-analyzer isotope ratio mass spectrometry (EA-IRMS) in **duplicate** for animal samples and **quadruplicate** for feed heterogeneous samples. Liquid samples (plasma and milk) can be analyzed if they are dried during 24h at room temperature within the open tin capsule used for EA-IRMS analysis. Results of natural ^{15}N abundance ($\delta^{15}N$) are expressed in delta units (‰) and analytical errors are usually around 0.1‰ for plasma samples, 0.20‰ for milk samples and 0.30‰ for feed samples. If feed ingredients are used instead of the whole TMR diet, the $\delta^{15}N_{diet}$ is calculated as the sum of feed ingredients weighted by their respective N content. For calculating $\Delta^{15}N_{animal\text{-}diet}$ the values of $\delta^{15}N_{diet}$ should be subtracted from $\delta^{15}N_{animal}$. The $\Delta^{15}N$ allows comparing animals fed different diets.

1.4 Advantages and limits of $\Delta^{15}N$ as a robust biomarker of feed efficiency in cattle

1.4.1 Advantages

- Better potential of $\Delta^{15}N$ vs milk or plasma urea for phenotyping purposes in cattle.
- $\Delta^{15}N$ values have been proven to reflect between-animal variability in feed efficiency or N use efficiency in ruminants. It has been shown that when $\Delta^{15}N$ values and N use efficiency are repeatable traits, the ability of this biomarker to predict between-animal variability in MNE is extremely good.
- $\Delta^{15}N$ values are stable across the day and do not show variability according to time of blood or milk sampling, no postprandial variation is thus expected.
- $\Delta^{15}N$ values do not reflect changes related to renal urea reabsorption and clearance rate. In contrast, they may reflect variation in some mechanisms related to N partitioning that are not caught by urea concentration in plasma or milk such as endogenous N losses and urea-N recycling.

1.4.2 Limits

- Analysis of $\Delta^{15}N$ is complex and needs a mass spectrometry apparatus and high technical skills, time consuming (maximum 30-40 samples/day) and relatively expensive (around 15-20 €/sample). Isotopic analysis at natural abundances are proposed by few laboratories worldwide or alternatively can be done in a collaborative framework with researchers of the Herbivore Research Unit at INRAE (contact: gonzalo.cantalapiedra@inrae.fr).
- When comparing dietary treatments, $\Delta^{15}N$ is very sensitive to inaccuracies of ^{15}N values in diets, which sometimes are very heterogeneous and not completely representative of what the animal really ate. Attention should be paid to the representativeness of diet samples and their homogeneity (ground on 0.5 mm rather than 1 mm).
- When shifting the dietary treatment, animal proteins need a relatively long time (minimum 4 weeks) to reach an isotopic equilibrium with the new diet. This time is thus recommended to be respected when comparing animals or diets in terms of feed efficiency.
- $\Delta^{15}N$ values are extremely sensitive to body weight loss. If animals are known to undergo body weight loss, this biomarker should not be used. In dairy cows, no prediction equations can be proposed when days in milk are lower than 50 since protein mobilization occurring before the milk peak strongly affect the ^{15}N signatures in animal proteins, perturbing the expected negative relationship between N use efficiency and $\Delta^{15}N$ values.
- Preliminary results suggest that the relationship between $\Delta^{15}N$ values and feed efficiency at the individual level could be dependent on the type of diets fed to animals with greater responses obtained with more energy dense diets promoting lower rumen protein balance.
- Preliminary results suggest that the relationship between $\Delta^{15}N$ values and feed efficiency at the individual level could be dependent on the type of breed, with greater responses obtained with late vs early maturing breeds.

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2 Faecal NIRS as proxy of total tract digestibility in cattle

2.1 Principle by which the proxy is related to the phenotype(s)

Near-infrared spectroscopy (NIR) is a commonly used technology for the management of livestock systems. Today, it is more and more used because of economic, environmental and regulatory reasons. In particular, it is used by industrial laboratories for the estimation of the chemical composition and digestibility of feedstuffs. Near-infrared spectroscopy quantifies the absorption of electromagnetic radiation in the near infrared region (700 to 2500 nm) when it interacts with the chemical bonds between the atoms of organic molecules **(Osborne and Fearn, 1988)**. The absorptions measured by NIR spectroscopy at different wavelengths correspond to overtones and combinations bands of vibrational modes involving organic matter chemical bonds of the sample **(Bertrand and Dufour, 1991)**. The collection of absorbance values at different wavelengths of near infrared region is called NIR spectra (NIRS). The absorbance values are therefore closely related to the amount of chemical bonds and thus to the chemical composition of the sample. Therefore, the NIR spectrum of a sample is unique and fully representative of it. For the quantification of the chemical composition from NIRS, it is required previously a calibration step that links the NIR spectrum to the results obtained by the laboratory for the reference methods. Faeces are the result of the digestion process of ingested feed by animals. Their chemical composition could be closely related to the digestibility of the diet **(Demarquilly et al., 1995)**. Therefore, NIR spectra of faeces can provide information on the digestive use of the diet and particularly to the organic matter digestibility (OMD), which is closely related to the energy value of the diet. Consequently, we hypothesised that faecal NIRS is related to OMD of the diet by animals. Near infrared spectroscopy of faeces has been successfully used for predicting OMD, in tropical conditions (**Boval et al., 2004**) but also in temperate conditions on lactating grazing dairy cows (**Decruyenaere et al., 2012**) and on cattle for fattening (**Jancewizcz et al., 2016**). However, the 2 last models have been developed for specific animals in a narrow range of diets and had limited data numbers, thus restricting their range of application.

2.2 Prediction equations

2.2.1 OM digestibility in cattle

In the SmartCow project, we collected 2 different data sets for predicting OMD: 1) faecal samples and values of in vivo OMD measured with the gold standard method (GSM; OMD_GSM); and 2) faecal samples and values of OMD measured indirectly using indigestible markers (OMD_M). These 2 data sets were managed separately because their combination was not relevant due to differences in the basical principle of the reference methods for OMD. Therefore, two different models of prediction were developed; one from the OMD_GSM values and the other one from OMD_M values.

In vivo OM digestibility measurement using with the GSM consists in 10-15 days of adaptation to the diet and 7-10 days of measures. During the measurement period, offers, refusals and the total faeces are daily collected and weighted before to be pooled on the period. Then, a representative sample of each of the 3 matrices is obtained, and after drying, samples are analysed for crude ash (550°C, 24h). Finally, the OMD is calculated as (OM ingested – OM excreted in faeces)/OM ingested described (**Mesgaran et al., 2020 in the Book of Method of SmartCow**). The standard error (RMSE) of the GSM was calculated from values issued from an experiment (**De la Torre et al., 2019**) where in vivo OMD was measured 2 consecutive times on 16 suckling cows fed 2 diets (100 % permanent grassland hay or based on 67% corn silage and 13% concentrate). The standard error (RMSEP) of the GSM for in vivo OMD was estimated as 0.0133.

When digestibility was estimated from markers (OMD_M), a marker was supplied to animals or an internal marker was chosen. A spot faeces sample was obtained, and the marker content administered or present in the diet and that present in the faeces was analysed. Organic matter digestibility was calculated according to Demarquilly et al., (1995). Three markers were used for estimation of OMD_D: Acid insoluble ash (AIA), chromic oxide and titanium oxide. The standard error of this method was not calculated.

The development of faecal NIRS models based on OMD_GSM dataset was carried out on 476 individual data from beef cows, lactating cows and males fed different diets. Research centres (INRAE from France, CRA-W from Belgium and Reading University from UK) shared individual faecal samples for NIRS analyses and corresponding values of OMD measured in vivo with the GSM. The faecal NIRS model based on OMD_M was performed using 693 individual data, provided by three research centres (IRTA from Spain, Aarhus University from Denmark and Agroscope from Switzerland).

All Faecal NIRS were obtained using an instrument NIRSystems 6500 equipped with a transport module (INRAE).

Calibration and validation of the two models (OMD_GSM and OMD_M) for the prediction of OMD were performed using R software according to the procedure reported in Delivrable 6.1 of this project**.**

- **Domain of validity:** The statistics associated with the domain of validity of the models are given in Table 1.

Table 1: Descriptive statistics of calibration and validation faecal samples from gold standard method-measured organic matter digestibility (OMD_GSM) and from marker-estimated organic matter digestibility (OMD_M).

	Calibration					Validation					
	N	Mean	Min	Max	Sd	N	Mean	Min	Max	Sd	Se
OMD_GSM	380	71.88	59.73	88.40	4.17	96	70.37	62.96	77.50	3.75	1.33
OMD_M	553	73.44	55.11	87.89	4.38	143	73.78	61.24	83.72	3.92	-

N: number of samples; Min: minimum value; Max: maximum value; Sd: standard deviation; Se standard error of gold standard method.

The statistics associated to the selected NIRS models for predicting OMD are given in Table 2.

Table 2. Performance of NIRS models for predicting organic matter digestibility measured *in vivo* using the GSM (OMD_GSM) or estimated by markers (OMD_M).

	RMSE	R^2	Bias	RMSE(c)
OMD_GSM	1.64	0.81	0.10	1.65
OMD_M	1.98	0.74	0.17	1.98

RMSE: standard error of prediction; R^2 coefficient of determination; RMSE(c): standard error of prediction corrected by bias;

- **Precision:** The RMSE for NIRS OMD_GSM prediction was 1.65 %. Consequently, the minimum detectable difference in OMD_GSM with the obtained NIRS model is 6.4% (CI 95%) meaning that predicting OMD_GSM in 2 different animals will allow to discriminate their OMD if they differ by 6.4%. Otherwise, the RMSE of the OMD measured by the GSM was 1.33. Consequently, the minimum detectable difference in OMD with the GSM is 5.2 %. Both minimum detectable differences are close confirming the good potential of faecal NIRS as a proxy for OMD prediction in cattle.
  For OMD_M, the RMSE for NIRS prediction was 1.98 %. The minimum detectable difference when the NIRS model is 7.8% (CI 95%).

- **Example of power analysis:** For the prediction error obtained for the OMD_GSM model, we can theoretically detect significant differences (CI 95%) between 2 groups of animals differing in 3 points of OMD if each group is composed of 5 animals. For detecting significant differences between two groups of animals differing in 2 points of OMD, it would be necessary to use 11 animals per group.
  For OMD_M predictions, 7 animals per group would be necessary for detecting significant differences between two groups of animals differing in 3 points of OMD_M, whereas 16 animals/group would be necessary for detecting significant differences between 2 groups of animals differing in 2 points of OMD.

2.3 What is needed for using faecal NIRS as a proxy of OM digestibility?

2.3.1 Samples

The OMD_GSM model has been built using pooled faecal samples from several days (at least 4). In order to get as closely as possible to the conditions which the models were obtained, we suggest to take spot samples from several days (n=4) according to the following protocol. Take individual fresh faeces samples (300 g spot sampling). It is recommended to sampling faeces by hand directly from the rectum. Samples can eventually be taken on the floor immediately after excretion, with a high attention to avoid contamination (urine, hay, grass, etc.) and regarding the identification of the specific animal. The samples can be taken at any time during the day.

After collection, put a thin layer of faeces (max. 2 cm height) in trays in order to avoid mould before drying at 60° during 72h. Pool the samples at the end of the last sampling period and then, faeces should be ground at 1 mm screen. Freezing samples is possible if they cannot be taken care of right away. The use of faecal samples dried according to other drying protocols (103°C, 80°C, freeze-dried) could be interesting, but its influence on the accuracy of the models would deserve to be analysed. The robustness of the model when it is applied on one spot faecal sample has not yet been tested but we hypothesized that model performances should not be very different from those obtained for the tested models.

2.3.2 Analyses

NIRS of faecal samples should be obtained placing the dry fecal sample on 50 mm diameter ring cup and scanned in the range of 400–2500 nm **in duplicate** using a Foss NIRSystems model 6500 scanning visible–NIR spectrometer. Users can obtain NIRS from different spectrometer devices. If the models are to be applied to NIRS obtained using other instruments, a standardization process is necessary. This process requires the collection of spectra of several samples by both instruments; the one used for collecting spectra used to develop the model and that used for obtaining the spectra of the samples to be predicted. Then, a mathematical correction should be calculated and applied to the spectra to be predicted (**Shenk and Westerhaus, 1991**). **Contact donato.andueza@inrae.fr for information on the standardization procedure.**

2.4 Advantages and limits of faecal NIRS as a robust proxy of OM digestibility

2.4.1 Advantages

- The accuracy of faecal NIRS for predicting OMD is close to that of GSM, which confirms that faecal NIRS is a robust proxy for prediction of OMD in both dairy and beef cattle. This is also a good alternative to the GSM using stalls, which are constraining for experimental animals (3R rules).
- **Collection of faeces**: 1 spot faecal sample per animal during at least 4 days is required any time during the day.
- **Sample conditioning**: processing of faecal samples (drying, grinding) can be subcontracted to a routine laboratory.
- **NIRS analyses:** low cost of the NIRS analyses when calibrated spectrophotometer device is available. To obtain the models or apply them to the faecal spectra acquired, **contact donato.andueza@inrae.fr**

2.4.2 Limits

- **Collection of faeces:** this can be done by waiting for the animal to excrete the faeces (waste of time) or by hand rectal sampling. Both sample collection methods need animal staff to restrain the animals and to collect samples. The collection of faeces samples on the floor is not recommended because it can be a source of error, but if it is a last resort, collection of faeces should be done immediately after excretion with a high attention to avoid contamination (urine, hay, grass, etc.) and regarding the identification of the specific animal.

- **Sample conditioning (drying, grinding):** this process step of the samples is time consuming. The development of methodology for direct analysis of fresh faeces in the field by NIRS (portable spectrometer) is in progress for new research recommendations.
- **No centralized routine laboratory exist to perform the NIRS analysis on faeces. Please contact donato.andueza@inrae.fr**
- **Missing data in the reference dataset**: young bulls, heifer and beef cows for OM digestibility are poorly represented in the SmartCow database. New reference data (OMD values and corresponding faecal NIRS) using standardization protocols for sampling and dataset management are required to improve robustness of the models. **For sharing data and contribute to increase the domain of validity of the models, please contact donato.andueza@inrae.fr**

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3 Faecal NIRS & milk MIRS as proxy of enteric methane emissions in cattle

3.1 Principle by which the proxy(ies) is related to the phenotype

The relevance to consider faecal near-infrared spectra (NIRS) to predict OMD has been investigated in a previous chapter. On another hand, eructed methane (CH_4) is consecutive to rumen fermentation process also related, among others, to the intake of OMD. Based on this, it seems relevant to investigate the feasibility to estimate eructed CH_4 from fecal NIRS.

Proxies related to milk composition, like milk fatty acid (MFA) and mid-infrared (MIR) spectra, seem to be a promising way to predict enteric CH_4 emissions because precursors for methanogenesis and de novo synthesis of MFA both arise in the rumen **(Negussie et al, 2017)**. Milk composition (fat, protein, lactose and urea contents), including some FA **(Soyeurt et al., 2011)**, can be determined routinely and at low cost by mid-infrared (MIR) spectroscopy in milk recording laboratories. Milk MIR spectra present a good potential as a proxy for prediction of CH_4 emissions in dairy cattle, especially when combined with animal characteristics such as lactation stage **(van Gastelen and Dijkstra, 2016)**, milk yield, parity and breed **(Vanlierde et al., 2020)**. This high throughput approach allows CH_4 production to be incorporated in dairy cow breeding programs.

3.2 Prediction equations

3.2.1 Faecal NIRS

In the SmartCow project, thanks to a strong collaborative network, several datasets combining CH_4 measurements and corresponding faecal NIR spectra (NIRS) have been shared and collected. Data related to dairy or beef cattle were managed separately. An important issue to combine datasets was the inconsistency of reference methods and sampling protocols used between them. As the main interest of this proxy is the estimation of CH_4 emitted by cattle without milk and considering the amount of data available for the moment, only the first model based on beef cattle is detailed here.

From the reference datasets available, a choice has been done to obtain the best compromise to have a sufficient amount of quality reference data with the same reference method, sampling protocols and a perspective of evolution of the model in the future with the collection of new reference data. Thus, CH_4 data collected with the GreenFeed system during 3 weeks and related to a faecal NIR spectra obtained from a sample collected at the end of the three considered weeks of CH_4 measurement have been considered (Table 3.1).

Table 3.1: Descriptive statistics of reference data considered for calibration for the first model to estimate enteric CH_4 emissions from faeces NIR spectra.

		CRA-W	INRAE
Breed		Belgian Blue, Dual purpose Belgian Blue	Charolais
Physiological stage		Calves, suckling cows, reformed cows	Heifers
N data		85	268
CH_4	Mean (g/d) ± SD	264 ± 47	207 ± 32
	Min - Max	94 - 377	127 - 320

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The developed models were performed using 346 measures and it was tested considering a four groups cross-validation procedure. This model presented a R^2 of calibration and cross-validation about 0.62 and 0.55 respectively and the RMSE for calibration and validation respectively were 26 and 29 g/d of CH_4. This model is a very first approach and will be improved with the acquisition and inclusion of new reference values. **If you have an opportunity to contribute, please contact donato.andueza@inrae.fr.** The reasonable error value is particularly interesting considering the very indirect aspect of this proxy.

3.2.2 Milk MIRS

As described in **Vanlierde et al. (2015, 2020)**, the equation is based on the milk mid infrared (MIR) spectral information combined with the days in milk information (DIM) to estimate individual CH_4 emissions. The last published model includes 1,089 reference data collected from 299 individual dairy cows in Belgium, Ireland, United Kingdom, France, Denmark, Switzerland and Germany with a mean of CH_4 (g/d) ± SD about 413 ± 102. Methane measurement were performed with respiration chambers and the SF_6 tracer techniques. A majority (82%) of the reference data are from Holstein cows, but the model also includes Jersey (6%), Brown Swiss (7%) and other punctual breeds (5%). The expected metabolic status of animals is included directly in the spectral information thanks to the use of a mathematical modification that takes into account the days in milk of animals. The model presented a R^2 of calibration and cross validation about 0.68 and 0.64 respectively, and errors of calibration and cross-validation about 58 and 61 g of CH_4 per day respectively. Description of experimental conditions used for developing prediction equations can be found here: **https://doi.org/10.1002/jsfa.109694**

At this stage, the inclusion of reference values obtained with the GreenFeed system in the previously mentioned model is not relevant due to differences between the basical principles of the reference techniques. However, as the relevance to consider milk MIR spectra as a proxy to estimate CH_4 emissions has been demonstrated and as several research teams collect data with GreenFeed system on a larger amount of animals and conditions, the development of a model based on CH_4 reference data collected with the GreenFeed system is under development. More especially the first methodological step investigated was about the best practical methodology to combine milk MIR spectra and the periodic CH_4 value obtained with GreenFeed system with the purpose of developing this predictive proxy **(Coppa et al., 2022).**

3.3 What is needed for using faecal NIRS or milk MIRS as proxies of enteric methane emissions in cattle?

3.3.1 Samples

Faecal samples:

A 300g (or more) spot sampling of individual fresh faeces is required. Sample can preferable be collected directly from the rectum or eventually, on the floor immediately after production, with a high attention to avoid contamination (urine, hay, grass, etc.) and regarding the identification of the specific animal. No particular attention needs to be paid about the moment of collection especially if animals receive diet with stable composition. If possible, collection of a sample from the first morning faeces is ideal. Samples need to be dried (60°C, 72h) by spreading the sample in a tray to have 1-2 cm of height maximum (avoiding moistures). Once dried, sample should be grinded (#1mm) before analyse of NIR spectra.

Collection of new reference data to upgrade the model: If you have the opportunity to perform CH_4 measurements on animals, please consider the collection of additional fecal samples during the period. You need to collect fecal sample with the protocol described in the previous paragraph. To ensure to possibility to merge your data to the existing datasets please respect the flowing timing of sampling. If you measure CH_4

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with respiration chambers or SF_6 technique, the faecal spot sample can be taken the same day than the measurement. If you are using the GreenFeed system for CH_4 measurement, the faecal spot sample should be taken at day 14 after the beginning of CH_4 measurement. If less than 20 visits of the Greenfeed system occur during this period by the animal, than consider a second sampling at day 21.

Milk samples:
A 40 mL of fresh milk sample representative of one milking or a sample representative of two consecutive milking (as performed in some milk recording scheme) and preserved at 4°C with 0.02 % bronopol. The lactation stage (days in milk) of the animal and the day of milk collection need to be known.

Collection of new reference data to upgrade the model: If you have the opportunity to perform CH_4 measurements on lactating dairy cows, please consider the collection of additional milk samples during the period to improve this proxy or validate it in these specific conditions. This is particularly interesting if your country is not yet included in the reference dataset described, if you perform CH_4 measurement on a different breed than Holstein (already well represented in classical conditions), if you are using innovative management techniques or specific diets which might not be included yet. To ensure the possibility to merge your data to the existing datasets please respect the flowing timing of sampling. If you measure CH_4 with respiration chambers or SF_6 technique, please take the same day than CH_4 measurement a representative sample of milk at each milking or one single milk sample including 50% of AM and 50% of PM milking. If you are using the GreenFeed system for CH_4 measurement, please take representative milk samples at each milking or one single milk sample including 50% of AM and 50% of PM milking, twice a week during at least 2 weeks. If less than 20 visits of the GreenFeed system occur during this period by the animal, than consider an additional week. In any case, preserve them at 4°C with 0.02 % bronopol before analyze by MIR spectrometry

3.3.2 Analyses

NIRS
NIR spectra should be obtained with a standardized apparatus. The standardization process requires the collection of spectra of several samples by both instruments; the one used for collecting spectra used to develop the model and that used for obtaining the spectra of the samples to be predicted. Then, a mathematical correction should be calculated and applied to the spectra to be predicted. **Please contact *donato.andueza@inrae.fr* to be informed on the standardization procedure.**

MIRS
MIR spectra should be obtained with a standardized apparatus. **Please contact c.grelet@cra.wallonie.be to be informed on the standardization procedure as established within the OptiMir protocol.**

3.4 Advantages and limits of faecal NIRS or milk MIRS as robust proxies of enteric CH4 emissions

3.4.1 Advantages

Faecal NIRS

- Faecal NIRS is a promising proxy for prediction of CH_4 emissions across individuals and diets in cattle especially useful for non-lactating cattle (beef, calf, heifers, dry dairy cows).
-

Milk MIRS

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- Already collected in routine (high throughput)
- Inclusion of data from several countries, breeds, herds' managements, diets, etc. in the reference dataset.
- Easily applicable and updating.
- Reasonable cost especially in comparison with reference methods to measure methane.
- Permits to perform large scale studies on farm (genetics, herds, regions)

3.4.2 Limits

Faecal NIRS

- As this proxy is still in very early stages of development, the robustness is low and the performances are highly related to the reference dataset. Moreover, no information about the sensibility of the model to various diets composition or additives is known for the moment.
- Missing reference data for CH4 emissions from faeces: other breeds, diets, physiological status, etc would strengthen robustness of the SmartCow model. **The inclusion of new reference data to cover this specific information is highly recommended (contact: donato.andueza@inrae.fr).**
- Standardization of the protocols for sampling and dataset management is needed.

Milk MIRS

- This proxy has a known error around 60 g/d of CH4. This is still very interesting to distinguish high and low CH4 emitters and to perform large scale of genetic studies. However, this might be not relevant to use only this proxy to estimate CH4 emissions during nutritional trials where low variations of CH4 emissions level are expected.
- If a specific breed, diet, environmental condition, etc. are not represented in the reference calibration set, the known statistics of the model regarding the prediction quality cannot be guaranteed, as it is extrapolation. **The inclusion of new reference data to cover this specific information is highly recommended (contact: a.vanlierde@cra.wallonie.be).**
- This proxy is based on the principle that amount of CH4 emissions as well as milk composition (and consequently, milk MIR spectra) are directly related to ruminal fermentations processes. If some additives are given to the animal and do not disturb this fundamental link, and with respect with the previous mentioned point, the predictions can be considered as usual. However, if the additive induce a reduction of the methane emission at the latest stages in the ruminal process, this will not impact the milk composition in the classical way and consequently the link between enteric CH4 emissions and milk composition is not maintained. In that case, the model will not be able to mark the impact of this specific additive on the enteric CH4 emission. Only the animal effect will be marked (related to the classical metabolic processes in the rumen).
- Dedicated to individual milk spectral and, even if applicable, no guarantee of the representativeness if applied on a bulk tank milk especially because DIM information needs to be considered but also because a bulk milk represents a several days mix of milk and cows will contribute to the total volume at different proportion in function of their parity and their lactation stage.

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