Dear PCI editor,

We sincerely thank you and the reviewers for the time they spent in reviewing our paper, and for their comments. We have answered to all comments, as you can see below. We hope that these changed will meet with your approval and allow the paper to be published.

Best regards,

Amélie Fischer, on behalf of all authors

Review by anonymous reviewer, 21 Apr 2021 09:37

I read and evaluated the article entitled "Feed efficiency of lactating Holstein cows is less reproducible when changing dietary starch and fibre concentrations than within diet over subsequent lactation stages" https://doi.org/10.1101/2021.02.10.430560. My main concern regards the experimental structure of the dairy cow trial, and I preferred to present it immediately to the authors. In particular, authors declared the main objective of the study was "…analysed the ability of lactating dairy cows to maintain their feed efficiency while changing the energy density of the diet by changing its concentration in starch and fibre", but they failed in verifying this because the adopted experimental design did not permit to properly separated the diet by period effects. I believe authors realized this when declared "The decrease in dietary net energy for lactation and in metabolizable protein was confounded with the increase of lactation stage as the experimentation was based on a sequential design. Therefore when effect of diet is mentioned here, it is confounded with the effect of lactation stage". Sincerely, this confused me because when an experimental design was planned, the main sources of variation in response parameters (at least the expected ones) should be properly controlled to avoid confusion about experimental terms of statistical model. Despite I found a lot of merit in other methodological approaches and general idea of this trial, I retained the experimental structure adopted did not permit to the authors to properly respond to the aim of the trial. Unfortunately, I am unable to suggest a possible solution for properly overcoming this – in my opinion – strong methodological issue.

Authors : We understand your point about the design. We agree that the present experimental design involves a confusion between time and diet effect, as we said in the discussion. However, we've discussed this limit in the section "Limits of the paper". As we said in this section, a crossover design is not perfect either because it can lead to an interaction between time and treatment, or to a remnant effect between time and treatment, which would imply the same confusion as the current paper's design. Moreover, A. Fischer et al. (other co-authors, other country) has also done a similar study where they did a crossover design and used a high starch/low NDF diet (starch = 27% diet DM, NDF = 29% diet DM) and a low starch/high NDF diet (starch = 13% diet DM, NDF = 37% diet DM). Their results are similar to the results and conclusions of the current studies that is, that feed efficiency is less reproducible across diets then within diet. This other paper is currently under review. For all these reasons, we can consider that our results are as valid as with a crossover design.

We have added the following sentences to the section "Limits of the study": "Another way to tackle confusion between lactation stage and treatment would have been to adopt a crossover design. However, this design can possibly lead to an interaction between time and

treatment, which is not quantifiable, or leads to a remnant effect of the first treatment. Moreover, a similar study was conducted by Fischer et al. where we used a cross-over design (paper under peer-review). The results and conclusions were similar to the current project. This supports the validity of the current paper."

Review by Ioannis Kaimakamis, 03 Oct 2021 17:15

The background section is cleraly and expalins the motivation and thw challenge of thw sudy. This research deals with a big issue on animal production and efficiency measurement. The experimental and the design of the research establishment well and with details. The variables selection are detailing with a strong references background. Also, the mathematical and statistical analyses are appropriate. The results section is clearly with a high value explainotary data and methods descrite.

The discusion and conclusion analysis are acceptable. The conclusions supported by the results . The references are appropriate and the main references are present.

I fully recommend the publicity of this research article.

> **Authors :** Thank you for your review and your comments.

Review by Angela Schwarm, 17 Dec 2021 21:49

I enjoyed reading this manuscript on the reproducibility of feed efficiency between an early lactation diet and a later lactation diet fed to 60 cows, and the repeatability of feed efficiency within diet. The authors discuss the sequential design and show that results are valid despite limitations. Two different indicators for feed efficiency were used, the CCC indicator and the errors indicator and the authors conclude that the former is more suitable to be used by animal breeders, whereas the latter is more suitable for farmers.

The title, abstract, and introduction could be improved to better reflect the content of the methods, results and discussion. The title could include the term repeatability (alternatively you could move graphs on repeatibility to the supplements) and the use of different indicators for assessing feed efficiency, e.g. Feed efficiency was less reproducible across diets than repeatable within diet using two indicators.

> **Authors :** we changed the title as you suggested, by including the notion of repeatability. However we preferred not to add the use of 2 indicators in the title not to confuse the reader. We prefer the reader to take home the main message of the paper, and for us the 2 indicators are not a key message. That's why we suggest the following title : "Feed efficiency of lactating Holstein cows was not as reproducible across diets as within diet over subsequent lactation stages."
>
> We have also changed the abstract so that it better reflects the content of the method, results and discussion.

Suggest to rephrase the last sentence of the abstract, e.g. to confirm on different ratios of forage to concentrate. During dry-off period/forage only the rank of feed efficiency can change because feeding costs are lowest.

> **Authors :** We changed the last sentence of the abstract as you suggested : "Those results have to be confirmed on diets having different forage to concentrate ratios to ensure that the least and most efficient cows will not change." We did not understand your suggestion "During dry-off period/forage only the rank of feed efficiency can change because feeding costs are lowest" do you want us to add this idea at the end of the abstract ?

Introduction, the motivation for the study and the research question could be more clearly presented by indicating the Fischer et al. reference as well as the two indicator methods. Reasoning for measurement of methane and carbon dioxide should be mentioned in the introduction.

> **Authors :** We changed the introduction, and especially the motivation for the study and the research question. As far as for methane and carbon dioxide, we considered them as "traits" used to describe the performance of the cows within both diets, along with production, body reserves and intake performance. For this reason, and because the study is focussing on feed efficiency repeatability, and not the relationship between feed efficiency and methane or carbon dioxide, we decided not to address this question in the introduction.
> The end of the introduction is now :
> « The main objective of the current study was therefore to check the ability of feed efficiency to be maintained across different diets. To achieve those objectives a trial was set up with lactating dairy cows that were fed with two diets. These diets differed in energy density, by lowering the starch concentration and increasing the fibre concentration of the diet. The feed efficiency was estimated within diet using the method developed in a previous paper (Fischer et al., 2018). The novelty of this paper was to estimate feed efficiency reproducibility across diets by combining two methods: the commonly used CCC in biology and the comparison of the error of reproducibility across diets with the error of repeatability within diet, as commonly used in metrology (ref de icar). Indeed, to estimate if FE is maintained across diets, its reproducibility across diets has to be compared to its repeatability within diet. If the reproducibility results are as good as the repeatability results within diet, then one can conclude that FE is as repeatable across diets as it is within diet. Opposedly if the reproducibility results are worse than repeatability within diet, then one can conclude that the ability of FE to be maintained across diets is not as good as within diet. As highlighted in the previous paragraph, a diet change could also lead to a change in cows sorting behaviour which could potentially affect feed efficiency. A second objective was therefore to check that the change in feed efficiency associated with diet change was not explained by differences in sorting behaviour. We therefore checked that feed efficiency was not associated with feed sorting behaviour by analysing the composition of each cow's diet refusals with near infrared spectroscopy. »

Materials and methods, more details should be provided for the methods and analysis:

-nutrients in feed were not analysed by wet chemistry but only assessed by NIR spectra. Suggest to indicate one or more references about the correlation of feed nutrient contents analysed with NIR spectra as compared to wet chemistry. As far as I remember e.g. fiber content might be less accurate than protein content in NIR compared to wet analyses. In wet chemistry, it is differentiated between NDF/ADF and ash corrected aNDFom/ADFom, I guess the NIR spectra would reflect rather the uncorrected NDF/ADF, but then comparisons of fiber contents between diets is restricted to those with similar ash contents. How much did the diets differ in ash content?

Authors : There must be a confusion between the part dedicated to the sorting behaviour (for which we use NIR spectra), and the part dedicated to feed intake, for which all analysis were done by wet chemistry, as explained in Material and Methods in part "Phenotypic Measurements". Indeed, all feeds were sampled, freeze-dried, ground, and then analysed for ash (muffle furnace), NDF and ADF (Van Soest method), fat content (ether extraction), starch (polarimetry), nitrogen concentration (Dumas method) to be able to calculate the nutritive values. This has already been detailed in the material and methods. To make it clearer, we added a subtitle in the « phenotypic measurements » part : « Individual feed intake and feed nutrient analysis » which includes the protocol for feed nutritive analysis and « Individual performance: milk, body weight and body condition, methane and carbon dioxide emissions. » which includes the monitoring of the performance.

As far as for the part of sorting behaviour which is based on NIR spectra : we don't actually need to know the chemistry composition of refusals or feed or diet. As the NIR spectra is the footprint of the sample, and that samples having different physical characteristics or chemical composition will also have different spectra, the comparison of our samples' NIR spectra will tell us if the samples have similar composition or not. All our analyses are here based on spectra comparison without estimating any chemical or physical characteristics. To make it clearer in the paper, we added a few sentences in the introduction about the use of NIR spectroscopy to estimate sample composition, as well as a few sentences in the material and methods.

In the introduction we have added : "Differences in feed composition were characterized by particle size differences in Dykier et al. (2020). However the method for particle size composition (Lammers et al., 1996; Kononoff et al., 2003) is heavy and time consuming. The advent of near-infrared (NIR) spectroscopy opens new ways to determine diet or feed compositions at high throughput. Indeed the NIR spectrum is sensitive to physical and chemical characteristics of the sample, and has therefore been used to determine nutritive value of feed, but also to discriminate samples according to their composition (De la Roza-Delagado et al., 2007, Li et al. 2007, Pérez-Marín et al., 2004, Xiccato et al., 2003)."

In the material and methods in part "checking for feed sorting behaviour" we have added : "Instead of determining each sample's feed composition, we used an indirect approach based on near-infra red spectroscopy. Indeed, we have seen in the background section of this paper that NIR spectroscopy can be used to differentiate samples differing in ingredients proportion. By definition, if the samples differ on a physical or chemical basis, their spectra will also be different. In this study, the differences in refusals composition and diets composition will be analysed through

their NIR spectra, without estimating or analysing their chemical or physical characteristics."

Table 1: -add organic matter content (ash was determined, so you should be able to calculate DMI-ash=OM); -is the amount of energy concentrate provided through the GreenFeed included in the energy concentrate listed in Table 1? Indicate in the running text as well (L135+) and state if concentrate feeder additional to GreenFeed was used or if a mixed ration was provided including the energy concentrate.

Authors : ok, we added OM in Table 1 as suggested. The diet composition distributed at the manger included all ingredients as described in part « Phenotypic measurements ; Individual feed intake and feed nutrient analysis ». The energy concentrate distributed at the Greenfeed was just a complement to the main diet which is used to attract and maintain each cow in the Greenfeed station to be sure that we would have enough measurements of methane and carbon dioxide. Therefore we added the following sentence to make it clearer in the manuscript:

"In addition to both diets, cows had access to a gas emissions monitoring system, the Greenfeed® (see "Individual performance: milk, body weight and body condition, methane and carbon dioxide emissions" section), which distributes small drops of energy concentrates to maintain the cow in the gas recording system. The amount of energy concentrates distributed per cow per day in the Greenfeed® station was added to the daily intake at the manger."

And in the table 1 footnote we've added : "The part of energy concentrate in the diet, as described here, includes the part of energy concentrates distributed at the Greenfeed® station."

-why was methane and carbon dioxide measured?, L207 section does not include CH4 energy loss

Authors : Methane and carbon dioxide have been measured because they are indicators of each cow's performance, such as milk production and composition, body weight, body weight change and body condition. We consider them as performance, such as milk production.

-Line 172 and carbon dioxide as well, not only methane

Authors : the only reason for us to add carbon dioxide was that it was also measured by the Greenfeed station and is also a greenhouse gas.

-Indicate maximum number of visits per day or give an average of number of visits per day to indicate accuracy of the methane production measured.

Authors : On average the cows visited the Greenfeed 2.2 /d (+/- 0.9). We have added this information in the material and methods.

As far as I can judge, the statistical analyses appears appropriate. The definition of significance $p<0.05$? is missing, add to the methods.

Authors : We've added the following sentence at the end of material & methods : "All statistical analysis were done with the significance level of 0.05 ($p \leq 0.05$).".

Results. Reviewers are asked to «check that raw data are available to the reader.», are the raw data available?

Authors : yes the raw data are available as well as all scripts used to analyse the data and estimate the results. This is mentioned at the far end of the paper in the part « Availability of data and material ».

Figure 4,5 S+F- diet defined in the figure legend as high starch-low fiber diet instead of high starch/energy-high protein as the acronym suggests. With metabolizable protein and net energy being only different by 10%, but starch and NDF being different by 19% and 16%, respectively, consider to use acronyms S+F- vs. S-F+ instead of S+F- vs. S-F+ to be consistent with manuscript title, introduction, discussion.

Authors : ok, we have changed it as suggested in the entire manuscript, tables and figures.

-L331, higher feed efficiency = higher CH4/DMI, cite other studies in the discussion which are in line with this observation.

Authors : the results in the current paper do not show that there is a high correlation between feed efficiency and CH4/DMI. Indeed, it shows that the change in randomRNEI when changing from diet S+F- to diet S-F+ was correlated to the change in CH4/DMI. That means that (randomRNEI in diet S+F- - randomRNEI diet S-F+) was correlated with (CH4/DMI diet S+F- - CH4/DMI S-F+). Here we are talking about the correlation between the difference between diets and not the correlation of the 2 variables within diet. This correlation within diet, as done in other papers (Fitzsimons et al., 2013; McDonnell et al., 2016; Flay et al., 2019; Olijhoek et al., 2017; Fischer et al., 2020) is not the purpose of the current paper. For this reason we prefer not to add more results and discussion about a topic which is not the aim of this paper.

We rephrased the sentences as follows to avoid any misunderstanding: “The change in randomRNEI induced by diet change (randomRNEI diet S+F- - randomRNEI diet S-F+) was negatively correlated with the change in methane yield, as per kg DMI, (CH4/DMI diet S+F- - CH4/DMI diet S-F+) with a Pearson correlation of - 0.31 ($p = 0.05$), but was neither significantly correlated with the change in methane production per day ($p = 0.12$) nor with the change in methane yield, as per kg milk ($p = 0.98$). This means that a cow that had a lower randomRNEI (higher feed efficiency) in diet S-F+, also had a higher methane yield per kg DMI in diet S-F+ than when fed with the S+F- diet, and conversely.”

To answer to your question about the correlation between CH4/DMI and feed efficiency, this correlation is not observed homogeneously in the literature: some see a higher CH4/DMI for higher feed efficiency (Fitzsimons et al., 2013; McDonnell et al., 2016; Flay et al., 2019; Fischer et al., 2020) but others see no correlation between both variables (Olijhoek et al., 2017). These studies differ in their experimental environments (breed, diet). Those environment differences may select different mechanisms involved in feed efficiency differences, and explains why the results are not homogeneous across studies about feed efficiency determinants.

Discussion. Suggest to rephrase the manuscript title, the heading of the first section of the discussion and the first sentence of the first section of the discussion to be less contradictive:

Manuscript title: Feed efficiency of lactating Holstein cows is less reproducible when changing dietary starch and fibre concentrations than within diet over subsequent lactation stages

Authors : Ok, we changed it to « Feed efficiency of lactating Holstein cows was not as repeatable across diets as within diet over subsequent lactation stages ».

Heading of first section of discussion: Feed Efficiency was nearly as reproducible across diets than repeatable within diet

Authors : Ok, we changed it to «Feed Efficiency was less reproducible across diets than within diet ».

First sentence in first section of discussion: Feed efficiency was less reproducible across diets than repeatable within diet …

Authors : Ok, we changed it to « Feed efficiency was less reproducible across diets than within diet ».

-when comparing the sub-period 2 S+F- with the sub-period 1 S-F+, the adaptation periods are not equally long, but 36 days for sub-period 2 S+F- and 23 days for sub-period 1 S-F+, please discuss.

Authors : usually, we consider that the time required for cows to adapt to a new diet that is significantly different from the current diet is about 2-3 weeks. That's why we chose 23 days when changing from diet S+F- to diet S-F+. Whether it is 36 or 23 days, it is more than required to let the cow adapt to its new diet. We added the following sentences to the "limits of the study" part in the discussion: "A last limit to the study is the length of the adaptation period between diet S+F- and S-F+. When comparing subperiod 2 of diet S+F- with subperiod 1 of diet S-F+, we compared two periods which had different length of adaptation period. Indeed the first had at least subperiod 1 of diet S+F- (36 days) whereas the second had 23 days. As we commonly consider that 2 to 3 weeks are enough to ensure that the cows are fully adapted to a new diet, we considered that the adaptation to the diets was achieved for the data used in the current study, and therefore we considered that the difference in length of the adaptation period did not influence the results.".

L420, where do you depict the cow's ranking in feed efficiency according to CCC? The cow's ranking 1 to 60 could be indicated in a supplemental table.

Authors : We used CCC as an indirect indicator of ranking. Cow's ranking was depicted with the CCC estimation, and is illustrated with the figure 2. We have added the ranking of the cows in the supplementary material in section "Availability of data and material ". We have changed the sentence in the discussion as follows : "However, as shown with the CCC and the cow's ranking (see section "Availability of data and material" for this supplementary material), the change in cow's ranking was similar when comparing between the two diets than when comparing within diet over subsequent lactation stages."

L506 was the difference in NDF (16%) too small to change milk fat contents? How big was the difference in NDF content between diets in other studies that found a difference in milk fat contents? Suggest to add to discussion.

Authors : The current study had a change in NDF representing 16% of the highest NDF. In the other studies this change was between 18% and 38%. Therefore the change in our study was slightly lower to those in the other studies, but similar to the

change in Karlsson et al. (18-20%). Moreover, the milk fat concentration was only significantly higher in Karlsson et al. for the diet having a change in 18% NDF. The other diets having more than 18% increase in NDF did not have a significant change in milk fat concentration. Therefore there is no clear relationship between increase in NDF and increase in milk fat concentration. We have added this to the discussion: "One could argue that our change in NDF between both diets, respectively of 16%, was too low compared to 18 to 38% in Boerman et al. (2015), Potts et al. (2015) and Karlsson et al. (2018) to see significant changes in milk fat concentrations. However, in Karlsson et al. (2018) only the lowest increase in NDF (18%) had a significant change in milk fat concentration; the other diets that had a higher change in NDF did not significantly increase the milk fat concentration. Therefore the increase in NDF may not systematically increase milk fat concentration."

L548, significant higher DMI and aD?

Authors : We changed it to be more accurate: "Higher methane emissions per day were observed when the change in dietary starch concentration was associated with significant differences in DMI (Bougouin et al., 2018) or significant differences in diet digestibility (Pirondini et al., 2015; Bougouin et al., 2018)."

L554, suggest to move the limitations of the study from the end to the start of the discussion.

Authors : we would like to maintain them at the end of the discussion because the most important take home message has to be put at the beginning of the discussion. Therefore, if you agree, we would prefer to keep the order of discussion as it is.

I do not want to claim that it is necessary to cite Pekka Huhtanen, but he did pioneering work in feed efficiency estimation. By this mean you would also include a reference from 2021 in your reference list.

Authors : we thank you for this interesting paper. This paper published by Huhtanen et al. in 2021 is about the use of residual CO2 as an indirect indicator of RFI in dairy cows. It is a very interesting and promising work published by Huhtanen et al., which will help the feed efficiency community for sure. However, as shown in other papers (Fitzsimons et al., 2013; McDonnell et al., 2016; Flay et al., 2019; Olijhoek et al., 2017; Fischer et al., 2020), the correlation between emissions and feed efficiency varies a lot, and highly depends on the diet. It is a promising work, which will need validation among other diets. Moreover, one would also not forget that the estimation of the residual CO2 indicator requires individual CO2 monitoring systems. Those systems are, so far, essentially based in research facilities. The Greenfeed station will help monitoring it in more farms, but it remains an expensive station which needs a high maintenance monitoring.

Wording/Formatting:

L293, exported -> partitioned

Authors : ok, we changed it as suggested.

L308, smaller? Rephrase

Authors : we changed "smaller" into "lower".

L524, hard -> difficult

Authors : ok, we changed it as suggested

Figure 2, indicate Figure 2a,b,c?!

Authors : ok, we changed it as suggested

Review by anonymous reviewer, 29 Dec 2021 07:32

The manuscript entitled "Feed efficiency of lactating Holstein cows is less reproducible when changing dietary starch and fiber concentrations than within diet over subsequent lactation stages" reports an interesting work on the evaluation of feed efficiency following the change of the ration in terms of starch level and fiber.
The work is well written with an in-depth description of the state of the art and the methodologies adopted.
Here are some details that deserve to be specified or deepened, for a better understanding of the work:

· Lanes 264-265: to estimate repeatability, state that you have used a fittied within each diet analysis of variance. It would be to provide a more detailed description of this statistical model.

Authors : To make it clearer, we have changed it into: "Repeatability was estimated within diet with an analysis of variance. For repeatability the model 3 below of analysis of variance was fitted once with the data of feed efficiency within diet S+F- to get the repeatability within diet S+F-, and once with the data of diet S-F+ to get the repeatability within diet S-F+."

· Lane 266: replace "die" with "diet".

Authors : ok, we changed it as suggested